(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
20 March 2003 (20.03.2003)

PCT

(10) International Publication Number
WO 03/023048 A2

(51) International Patent Classification[7]: C12Q

(21) International Application Number: PCT/US02/28410

(22) International Filing Date:
6 September 2002 (06.09.2002)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
60/317,653 6 September 2001 (06.09.2001) US

(63) Related by continuation (CON) or continuation-in-part (CIP) to earlier application:
US 60/317,653 (CIP)
Filed on 6 September 2001 (06.09.2001)

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(81) Designated States *(national)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, OM, PH, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, YU, ZA, ZM, ZW.

(84) Designated States *(regional)*: ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, SK, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:
— *without international search report and to be republished upon receipt of that report*

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

WO 03/023048 A2

(54) Title: METHODS FOR DIAGNOSING AND TREATING DISEASES AND CONDITIONS ASSOCIATED WITH PROTEIN KINASE Cλ

(57) Abstract: The invention provides methods of diagnosing diseases and conditions associated with PKCλ, methods for identifying compounds that can be used to treat or to prevent such diseases and conditions, and methods of using these compounds to treat or to prevent such diseases and conditions. Also provided in the invention are animal model systems that can be used in screening methods.

METHODS FOR DIAGNOSING AND TREATING DISEASES AND CONDITIONS ASSOCIATED WITH PROTEIN KINASE C λ

Field of the Invention

This invention relates to methods for diagnosing and treating diseases and conditions associated with Protein Kinase C λ.

Background of the Invention

The processes by which organs acquire global structures and patterns during development are highly complex, and likely involve multiple, overlapping biochemical pathways. In the vertebrate heart, for example, the first key visible step in this process is chamber morphogenesis, involving the fashioning of the atrium and the ventricle. Proper orientation of these two functionally distinct contractile units is required for unidirectional blood flow, which begins with the first heartbeat of an organism. Properly formed chambers thereafter are the substrates upon which further heart development is superimposed.

Over recent years, much has been learned about the molecular mechanisms that are responsible for the acquisition of characteristic atrial and ventricular cell fates (Fishman et al., Development 124:2099-2117, 1997; Srivastava et al., Nature 407:221-226, 2000). However, both embryological and molecular steps that fashion the higher order structures of these chambers have proven to be more elusive because, in part, unlike cell fate decisions, these steps can be studied meaningfully only in living organisms. The zebrafish, *Danio rerio*, is a convenient organism to use in genetic and biochemical analyses of development. It has an accessible and transparent embryo, allowing direct observation of organ function from the earliest stages of development, has a short generation time, and is fecund.

Summary of the Invention

The invention provides diagnostic, drug screening, and therapeutic methods that are based on the observation that a mutation, designated the "*heart and soul* (*has*)"

mutation, in the zebrafish Protein Kinase C λ (PKCλ) gene, as well as a small molecule identified in a chemical screen in zebrafish, concentramide, cause abnormal heart growth and development.

In a first aspect, the invention provides a method of determining whether a test subject (e.g., a mammal, such as a human) has or is at risk of developing a disease or condition related to PKCλ (e.g., a disease or condition of the heart; also see below). This method involves analyzing a nucleic acid molecule of a sample from the test subject to determine whether the test subject has a mutation (e.g., the *has* mutation; see below) in a gene encoding PKCλ. The presence of such a mutation indicates that the test subject has or is at risk of developing a disease related to PKCλ. This method can also involve the step of using nucleic acid molecule primers specific for a gene encoding PKCλ for nucleic acid molecule amplification of the gene by the polymerase chain reaction. It can further involve sequencing a nucleic acid molecule encoding PKCλ from a test subject.

In a second aspect, the invention provides a method for identifying compounds that can be used to treat or prevent a disease or condition associated with PKCλ, or in the preparation of a medicament for use in such methods. This method involves contacting an organism (e.g., a zebrafish) having a mutation in a PKCλ gene (e.g., the *heart and soul* mutation), and having a phenotype characteristic of such a disease or condition, with the compound, and determining the effect of the compound on the phenotype. Detection of an improvement in the phenotype indicates the identification of a compound that can be used to treat or prevent the disease or condition. In a variation of this method, the organism, with or without a mutation in the PKCλ gene (e.g., the *has* mutation), is contacted with a candidate compound in the presence of concentramide.

In a third aspect, the invention provides a method of treating or preventing a disease or condition related to PKCλ in a patient (e.g., a patient having a mutation (e.g., the *heart and soul* mutation) in a PKCλ gene), involving administering to the patient a compound identified using the method described above. Also included in the invention is the use of such compounds in the treatment or prevention of such diseases or conditions, as well as the use of these compounds in the preparation of medicaments for such treatment or prevention.

In a fourth aspect, the invention provides an additional method of treating or preventing a disease or condition related to PKCλ in a patient. This method involves administering to the patient a functional PKCλ protein or a nucleic acid molecule (in,

e.g., an expression vector) encoding the protein. Also included in the invention is the use of such proteins or nucleic acid molecules in the treatment or prevention of such diseases or conditions, as well as the use of these proteins or nucleic acid molecules in the preparation of medicaments for such treatment or prevention.

In a fifth aspect, the invention includes a substantially pure zebrafish PKCλ polypeptide. This polypeptide can include or consist essentially of, for example, an amino acid sequence that is substantially identical to the amino acid sequence of SEQ ID NO:2. The invention also includes variants of these polypeptides that include sequences that are at least 75%, 85%, or 95% identical to the sequences of these polypeptides, and which have PKCλ activity or otherwise are characteristic of the diseases and conditions mentioned elsewhere herein. Fragments of these polypeptides are also included in the invention. For example, fragments that include any of the different domains of PKCλ, in varying combinations, are included.

In a sixth aspect, the invention provides an isolated nucleic acid molecule (e.g., a DNA molecule) including a sequence encoding a zebrafish PKCλ polypeptide. This nucleic acid molecule can encode a polypeptide including or consisting essentially of an amino sequence that is substantially identical to the amino acid sequence of SEQ ID NO:2. The invention also includes nucleic acid molecules that hybridize to the complement of SEQ ID NO:1 under highly stringent conditions and encode polypeptides that have PKCλ activity or otherwise are characteristic of the diseases and conditions mentioned elsewhere herein.

In a seventh aspect, the invention provides a vector including the nucleic acid molecule described above.

In an eighth aspect, the invention includes a cell including the vector described above.

In a ninth aspect, the invention provides a non-human transgenic animal (e.g., a zebrafish or a mouse) including the nucleic acid molecule described above.

In a tenth aspect, the invention provides a non-human animal having a knockout mutation in one or both alleles encoding a PKCλ polypeptide.

In an eleventh aspect, the invention includes a cell from the non-human knockout animal described above.

In a twelfth aspect, the invention includes a non-human transgenic animal (e.g., a zebrafish) including a nucleic acid molecule encoding a mutant PKCλ polypeptide, e.g., a polypeptide having the *heart and soul* mutation.

In a thirteenth aspect, the invention provides an antibody that specifically binds to a PKCλ polypeptide.

By "polypeptide" or "polypeptide fragment" is meant a chain of two or more (e.g., 10, 15, 20, 30, 50, 100, or 200, or more) amino acids, regardless of any post-translational modification (e.g., glycosylation or phosphorylation), constituting all or part of a naturally or non-naturally occurring polypeptide. By "post-translational modification" is meant any change to a polypeptide or polypeptide fragment during or after synthesis. Post-translational modifications can be produced naturally (such as during synthesis within a cell) or generated artificially (such as by recombinant or chemical means). A "protein" can be made up of one or more polypeptides.

By "Protein Kinase C λ protein," "Protein Kinase C λ polypeptide," "PKCλ protein," or "PKCλ polypeptide" is meant a polypeptide that has at least 45%, preferably at least 60%, more preferably at least 75%, and most preferably at least 90% amino acid sequence identity to the sequence of a human (SEQ ID NO:5) or a zebrafish (SEQ ID NO:2) PKCλ polypeptide. Polypeptide products from splice variants of PKCλ gene sequences and PKCλ genes containing mutations are also included in this definition. A PKCλ polypeptide as defined herein plays a role in heart development, modeling, and function. It can be used as a marker of diseases and conditions associated with PKCλ, such as heart disease (also see below).

By a "Protein Kinase C λ nucleic acid molecule" or "PKCλ nucleic acid molecule" is meant a nucleic acid molecule, such as a genomic DNA, cDNA, or RNA (e.g., mRNA) molecule, that encodes a PKCλ protein (e.g., a human (encoded by SEQ ID NO:4) or a zebrafish (encoded by SEQ ID NOs:1 or 3) PKCλ protein), a PKCλ polypeptide, or a portion thereof, as defined above. A mutation in a PKCλ nucleic acid molecule can be characterized, for example, by the insertion of a premature stop codon anywhere in the PKCλ gene. For example, codon R515 can be changed to a stop codon (CGA to TGA), or codon W519 can be changed to a stop codon (TGG to TAG). In addition to this zebrafish Protein Kinase C λ mutation (hereinafter referred to as "the *heart and soul* mutation"), the invention includes any mutation that results in aberrant PKCλ protein production or function, including, only as examples, null mutations and

additional mutations causing truncations. The truncations can be carboxyl terminal truncations in which the carboxyl terminal half of the protein (or a portion thereof) is not produced. For example, at least 10, 25, 50, 70, 75, 100, 150, 200, or 250 amino acids of the carboxyl terminal half of the protein can be absent.

The term "identity" is used herein to describe the relationship of the sequence of a particular nucleic acid molecule or polypeptide to the sequence of a reference molecule of the same type. For example, if a polypeptide or a nucleic acid molecule has the same amino acid or nucleotide residue at a given position, compared to a reference molecule to which it is aligned, there is said to be "identity" at that position. The level of sequence identity of a nucleic acid molecule or a polypeptide to a reference molecule is typically measured using sequence analysis software with the default parameters specified therein, such as the introduction of gaps to achieve an optimal alignment (e.g., Sequence Analysis Software Package of the Genetics Computer Group, University of Wisconsin Biotechnology Center, 1710 University Avenue, Madison, WI 53705, BLAST, or PILEUP/PRETTYBOX programs). These software programs match identical or similar sequences by assigning degrees of identity to various substitutions, deletions, or other modifications. Conservative substitutions typically include substitutions within the following groups: glycine, alanine, valine, isoleucine, and leucine; aspartic acid, glutamic acid, asparagine, and glutamine; serine and threonine; lysine and arginine; and phenylalanine and tyrosine.

A nucleic acid molecule or polypeptide is said to be "substantially identical" to a reference molecule if it exhibits, over its entire length, at least 51%, preferably at least 55%, 60%, or 65%, and most preferably 75%, 85%, 90%, or 95% identity to the sequence of the reference molecule. For polypeptides, the length of comparison sequences is at least 16 amino acids, preferably at least 20 amino acids, more preferably at least 25 amino acids, and most preferably at least 35 amino acids. For nucleic acid molecules, the length of comparison sequences is at least 50 nucleotides, preferably at least 60 nucleotides, more preferably at least 75 nucleotides, and most preferably at least 110 nucleotides. Of course, the length of comparison can be any length up to and including full length.

A PKCλ nucleic acid molecule or a PKCλ polypeptide is "analyzed" or subject to "analysis" if a test procedure is carried out on it that allows the determination of its biological activity or whether it is wild type or mutated. For example, one can analyze

the PKCλ genes of an animal (e.g., a human or a zebrafish) by amplifying genomic DNA of the animal using the polymerase chain reaction, and then determining whether the amplified DNA contains a mutation, for example, the *heart and soul* mutation, by, e.g., nucleotide sequence or restriction fragment analysis.

By "probe" or "primer" is meant a single-stranded DNA or RNA molecule of defined sequence that can base pair to a second DNA or RNA molecule that contains a complementary sequence (a "target"). The stability of the resulting hybrid depends upon the extent of the base pairing that occurs. This stability is affected by parameters such as the degree of complementarity between the probe and target molecule, and the degree of stringency of the hybridization conditions. The degree of hybridization stringency is affected by parameters such as the temperature, salt concentration, and concentration of organic molecules, such as formamide, and is determined by methods that are well known to those skilled in the art. Probes or primers specific for PKCλ nucleic acid molecules, preferably, have greater than 45% sequence identity, more preferably at least 55-75% sequence identity, still more preferably at least 75-85% sequence identity, yet more preferably at least 85-99% sequence identity, and most preferably 100% sequence identity to the sequences of human (SEQ ID NO:4) or zebrafish (SEQ ID NOs:1 and 3) PKCλ genes.

Probes can be detectably labeled, either radioactively or non-radioactively, by methods that are well known to those skilled in the art. Probes can be used for methods involving nucleic acid hybridization, such as nucleic acid sequencing, nucleic acid amplification by the polymerase chain reaction, single stranded conformational polymorphism (SSCP) analysis, restriction fragment polymorphism (RFLP) analysis, Southern hybridization, northern hybridization, *in situ* hybridization, electrophoretic mobility shift assay (EMSA), and other methods that are well known to those skilled in the art.

A molecule, e.g., an oligonucleotide probe or primer, a gene or fragment thereof, a cDNA molecule, a polypeptide, or an antibody, can be said to be "detectably-labeled" if it is marked in such a way that its presence can be directly identified in a sample. Methods for detectably labeling molecules are well known in the art and include, without limitation, radioactive labeling (e.g., with an isotope, such as ^{32}P or ^{35}S) and nonradioactive labeling (e.g., with a fluorescent label, such as fluorescein).

By a "substantially pure polypeptide" is meant a polypeptide (or a fragment thereof) that has been separated from proteins and organic molecules that naturally accompany it. Typically, a polypeptide is substantially pure when it is at least 60%, by weight, free from the proteins and naturally occurring organic molecules with which it is naturally associated. Preferably, the polypeptide is a PKCλ polypeptide that is at least 75%, more preferably at least 90%, and most preferably at least 99%, by weight, pure. A substantially pure PKCλ polypeptide can be obtained, for example, by extraction from a natural source, by expression of a recombinant nucleic acid molecule encoding a PKCλ polypeptide, or by chemical synthesis. Purity can be measured by any appropriate method, e.g., by column chromatography, polyacrylamide gel electrophoresis, or HPLC analysis.

A polypeptide is substantially free of naturally associated components when it is separated from those proteins and organic molecules that accompany it in its natural state. Thus, a protein that is chemically synthesized or produced in a cellular system that is different from the cell in which it is naturally produced is substantially free from its naturally associated components. Accordingly, substantially pure polypeptides not only include those that are derived from eukaryotic organisms, but also those synthesized in *E. coli*, other prokaryotes, or in other such systems.

By "isolated nucleic acid molecule" is meant a nucleic acid molecule that is removed from the environment in which it naturally occurs. For example, a naturally-occurring nucleic acid molecule present in the genome of cell or as part of a gene bank is not isolated, but the same molecule, separated from the remaining part of the genome, as a result of, e.g., a cloning event (amplification), is "isolated." Typically, an isolated nucleic acid molecule is free from nucleic acid regions (e.g., coding regions) with which it is immediately contiguous, at the 5' or 3' ends, in the naturally occurring genome. Such isolated nucleic acid molecules can be part of a vector or a composition and still be isolated, as such a vector or composition is not part of its natural environment.

An antibody is said to "specifically bind" to a polypeptide if it recognizes and binds to the polypeptide (e.g., a PKCλ polypeptide), but does not substantially recognize and bind to other molecules (e.g., non-PKCλ-related polypeptides) in a sample, e.g., a biological sample, which naturally includes the polypeptide.

By "high stringency conditions" is meant conditions that allow hybridization comparable with the hybridization that occurs using a DNA probe of at least 100, e.g., 200, 350, or 500, nucleotides in length, in a buffer containing 0.5 M $NaHPO_4$, pH 7.2, 7% SDS, 1 mM EDTA, and 1% BSA (fraction V), at a temperature of 65°C, or a buffer containing 48% formamide, 4.8 x SSC, 0.2 M Tris-Cl, pH 7.6, 1 x Denhardt's solution, 10% dextran sulfate, and 0.1% SDS, at a temperature of 42°C. (These are typical conditions for high stringency northern or Southern hybridizations.) High stringency hybridization is also relied upon for the success of numerous techniques routinely performed by molecular biologists, such as high stringency PCR, DNA sequencing, single strand conformational polymorphism analysis, and *in situ* hybridization. In contrast to northern and Southern hybridizations, these techniques are usually performed with relatively short probes (e.g., usually 16 nucleotides or longer for PCR or sequencing, and 40 nucleotides or longer for *in situ* hybridization). The high stringency conditions used in these techniques are well known to those skilled in the art of molecular biology, and examples of them can be found, for example, in Ausubel et al., Current Protocols in Molecular Biology, John Wiley & Sons, New York, NY, 1998, which is hereby incorporated by reference.

By "sample" is meant a tissue biopsy, amniotic fluid, cell, blood, serum, urine, stool, or other specimen obtained from a patient or a test subject. The sample can be analyzed to detect a mutation in a PKCλ gene, or expression levels of a PKCλ gene, by methods that are known in the art. For example, methods such as sequencing, single-strand conformational polymorphism (SSCP) analysis, or restriction fragment length polymorphism (RFLP) analysis of PCR products derived from a patient sample can be used to detect a mutation in a PKCλ gene; ELISA and other immunoassays can be used to measure levels of a PKCλ polypeptide; and PCR can be used to measure the level of a PKCλ nucleic acid molecule.

By "Protein Kinase C λ-related disease," "PKCλ-related disease," "Protein Kinase C λ-related condition," or "PKC λ-related condition" is meant a disease or condition that results from inappropriately high or low expression of a PKCλ gene, or a mutation in a PKCλ gene (including control sequences, such as promoters) that alters the biological activity of a PKCλ nucleic acid molecule or polypeptide. PKCλ-related diseases and conditions can arise in any tissue in which PKCλ is expressed during

prenatal or post-natal life. PKCλ-related diseases and conditions can include diseases or conditions of the heart or cancer (also see below).

The invention provides several advantages. For example, using the diagnostic methods of the invention it is possible to detect an increased likelihood of diseases or conditions associated with PKCλ, such as diseases of the heart or cancer, in a patient, so that appropriate intervention can be instituted before any symptoms occur. This may be useful, for example, with patients in high-risk groups for such diseases or conditions. Also, the diagnostic methods of the invention facilitate determination of the etiology of such an existing disease or condition in a patient, so that an appropriate approach to treatment can be selected. In addition, the screening methods of the invention can be used to identify compounds that can be used to treat or to prevent these diseases or conditions. The invention can also be used to treat diseases or conditions (e.g., organ failure, such as heart or kidney failure) for which, prior to the invention, the only treatment was organ transplantation, which is limited by the availability of donor organs and the possibility of organ rejection.

Other features and advantages of the invention will be apparent from the following detailed description, the drawings, and the claims.

Brief Description of the Drawings

Fig. 1A is a schematic representation of the structure of a small molecule, concentramide, that alters heart patterning.

Fig. 1B is a lateral view of the mushroom-shaped heart of a live, concentramide-treated embryo 30 hpf. The atrium is indicated with **A**, and the ventricle with **V**.

Fig. 1C is a schematic representation of a timecourse of concentramide effectiveness. Black bars indicate the developmental time periods during which groups of embryos were immersed in water containing concentramide. An "x" indicates that treatment during the indicated time period alters the wild-type brain or heart phenotypes. An "o" indicates that the wild-type phenotype was observed. Blue and pink boxes mark the critical periods for development of the brain and heart phenotypes, respectively.

Fig. 2 shows that hearts from *has* mutant embryos phenocopy hearts from concentramide-treated embryos. *In situ* hybridization was performed with wild-type (Figs. 2A-2C), concentramide-treated (Figs. 2D-2F), and *has* (Figs. 2G-2I) embryos. The expression pattern of cardiac myosin light chain 2 (cmlc2) is shown for embryos 24

hpf (Figs. 2A, 2D, and 2G) and 30 hpf (Figs. 2B, 2E, and 2H). The relative locations of atrium (A) and ventricle (V) were confirmed by 7 μm sagital sections of embryos in which the ventricle was prestained blue by *in situ* hybridization to ventricle-specific myosin heavy chain (vmhc), followed by staining of the atrium brown with the atrium-specific antibody S46 (Figs. 2C, 2F, and 2I). The view is dorsal, anterior up in Figs. 2A, 2D, and 2G. The view is lateral, anterior to the left in all other frames.

Fig. 3A is a map of the *has* interval with genomic structure of the zebrafish PKCλ gene. YAC and BAC clones are indicated by addresses beginning with "y" and "b." The BAC clone 23c14 was sequenced to determine the entire genomic structure of the *has* gene. From the partial sequence of the BACs listed, a preliminary transcript map of the region was determined (see Table 1). The zebrafish PKCλ gene comprises 18 exons represented by vertical lines. The site of the mutations associated with the m129 and m567 alleles is indicated with an asterisk.

Fig. 3B is an anti-PKCλ western blot of protein extracts from wild-type embryos (WT), *has* mutant embryos (m567 -/-), and siblings of *has* mutant embryos (m567 +/+ and +/-).

Figs. 3C-3E show that antisense disruption of PKCλ expression phenocopies the *has* mutation. Wild-type embryos (3C), *has* embryos (3D), and wild-type embryos injected with a PKCλ antisense morpholino oligomer (3E) were photographed live 2 days postfertilization.

Fig. 4 shows that PKCλ is required for lamination, cell polarity, and epithelial cell-cell interaction in the retina. Transverse 5 μm sections of wild-type (Figs. 4A-4B), concentramide-treated (Figs. 4C-4D), and *has* (Figs. 4E-4F) embryos were stained with hematoxylin-eosin 5 days postfertilization (Figs. 4A, 4C, and 4E) or with dapi 30 hpf (Figs. 4B, 4D, and 4F). Arrowheads indicate mitotic nuclei. Zonula occludens-1 localization in the retina is shown by 5 μm transverse sections following staining of wild-type (Fig. 4G) or *has* (Fig. 4H) embryos with an anti-ZO-1 antibody.

Fig. 5 shows the effects of PKCλ inactivation and concentramide treatment on polarity of the zebrafish kidney and the *C. elegans* embryo. An apical kidney marker (3G8) was used to stain kidneys of wild-type (Fig. 5A), concentramide-treated (Fig. 5B), and *has* (Fig. 5C) embryos. Transverse 2 μm sections of the pronephric duct are shown. Figs. 5D and 5E, *C. elegans* strain KK871, a stable expresser of a par2:GFP fusion

protein, was treated with 34 μM concentramide and allowed to develop at room temperature. Nomarski (Fig. 5D) and fluorescence (Fig. 5E) microscopy were used to visualize the asymmetry of division and par2:GFP localization after the first cell division. Posterior is to the left.

Fig. 6 shows alterations in anterior-posterior patterning after treatment with concentramide. Figs. 6A-6C, *In situ* hybridization was used to show Pax2.1 expression in untreated (Fig. 6A) and concentramide-treated (Fig. 6B) 18-somite embryos. The expression patterns have been false-colored blue for untreated embryos and red for concentramide-treated embryos. Fig. 6C shows an overlay of the images from Figs. 6A and 6B. Arrowheads indicate areas of Pax2.1 expression at the midbrain-hindbrain boundary and in the otic placodes. The view is lateral, anterior to the left in Figs. 6A-6C. Fig. 6D, The distance between the anterior edge of the heart field, as defined by cmlc2 *in situ* staining, and the rostral extreme of the zebrafish embryo was measured in wild-type (WT), concentramide-treated (conc.), and *has* embryos at the 18-somite stage. Error bars represent standard error.

Fig. 7 shows the order of anterior and posterior heart field fusion. Dorsal views of cmlc2 expression at the16-somite (Figs. 7A-7C) and 18-somite (Figs. 7D-7F) stages. Expression patterns for wild-type (Fig. 7A and Fig. 7D), concentramide-treated (Fig. 7B and Fig. 7E), and *has* (Fig. 7C and 7F) embryos are shown. Anterior is up.

Fig. 8 is a schematic representation of a model for chamber patterning in the zebrafish heart. Normally, the bilateral primordia of the heart field converge and fuse first at the posterior end, followed by the anterior end to form a cone. The cone then rotates to orient atrial precursors toward the anterior and ventricular precursors toward the posterior in an extended heart tube. In concentramide-treated and *has* mutant embryos, the fusion order of the ends of the heart field is reversed, proceeding from the anterior to the posterior end. Rotation of the cone is blocked, preventing formation of the heart tube and causing the concentric heart chamber phenotype. Presumptive atrial precursor cells are colored red, ventricular precursor cells are colored blue. Views are dorsal; anterior is up.

Detailed Description

The invention provides methods of diagnosing, preventing, and treating diseases and conditions associated with PKCλ, such as diseases or conditions of the heart (also

see below), and screening methods for identifying compounds that can be used to treat or to prevent such diseases and conditions. In particular, we have identified a small molecule, concentramide, and a genetic mutation, *heart-and-soul* (*has*), which disrupt the earliest heart. Both cause the ventricle to form within the atrium. We show here that the *has* gene encodes an atypical Protein Kinase C, Protein Kinase C λ (PKCλ). The *has* mutation results in the disruption of epithelial cell-cell interactions in a broad range of tissues. Concentramide does not disrupt epithelial cell interactions but, rather, shifts the converging heart field of developing embryos rostrally. What is shared between the effects of concentramide and *has* is a reversal of the order of fusion of the anterior and posterior ends of the heart field.

The diagnostic methods of the invention thus involve detection of mutations in genes encoding PKCλ proteins, while the compound identification methods involve screening for compounds that affect the phenotype of organisms having mutations in genes encoding PKCλ or other models of appropriate diseases and conditions. The compound identification methods can also involve screening of candidate compounds in the presence of concentramide, using organisms with or without a PKCλ mutation (e.g., the *has* mutation). Compounds identified in this manner, as well as PKCλ genes and proteins themselves, can be used in methods to treat or prevent diseases and conditions associated with PKCλ. Compounds, antisense molecules, and antibodies that are found to inhibit PKCλ function can also be used to prevent or treat cancer.

The invention also provides animal model systems (e.g., zebrafish having mutations (e.g., the *heart and soul* mutation) in PKCλ genes, or mice (or other animals) having such mutations) that can be used in the screening methods mentioned above, as well as the PKCλ protein, and genes encoding this protein. Also included in the invention are genes encoding mutant zebrafish PKCλ proteins (e.g., genes having the *heart and soul* mutation) and proteins encoded by these genes. Antibodies that specifically bind to these proteins (wild type or mutant) are also included in the invention.

The diagnostic, screening, and therapeutic methods of the invention, as well as the animal model systems, proteins, and genes of the invention, are described further, as follows, after a brief description of diseases and conditions associated with PKCλ, which can be diagnosed, prevented, or treated according to the invention.

PKCλ-Associated Diseases or Conditions

Abnormalities in PKCλ genes or proteins can be associated with any of a wide variety of diseases or conditions, all of which can thus be diagnosed, prevented, or treated using the methods of the invention. For example, as discussed above, the *heart and soul* mutation in zebrafish is characterized by abnormal heart growth and development. Thus, detection of abnormalities in PKCλ genes or their expression can be used in methods to diagnose, or to monitor the treatment or development of, diseases or conditions of heart. In addition, compounds that are identified in the screening methods described herein, as well as PKCλ nucleic acid molecules, proteins, and antibodies themselves, can be used in methods to prevent or treat such diseases or conditions.

Specific examples of diseases or conditions of the heart that can be diagnosed, prevented, or treated according to the invention include congenital defects that result in heart malformation. These include congenital defects, such as Ebstein anomaly, which results in abnormalities of the tricuspid valve, as well as isomerism defects, which are characterized by a wide variety of abnormalities in the asymmetrical arrangement of particular organs, such as the heart, organs of the digestive tract, and the spleen, that normally occurs during development.

In right isomerism sequence, for example, which is also known as asplenia syndrome, Ivemark syndrome, and right atrial isomerism, the right side structures of the heart are duplicated on the left side of the heart, and the spleen is absent. This condition can lead to very complex and severe heart defects, such as atrioventricular septal defect (AVSD). In contrast, in left isomerism sequence, which is also known as polysplenia syndrome, the left side heart structures are duplicated and multiple small spleens may be present. This condition can lead to heart defects as well, such as heart block, which results in a slow heart beat, atrial septal defect, which is characterized by a hole between the top two heart chambers, and AVSD. With both types of isomerisms, twisting of the bowel or intestinal obstruction may result, due to the incorrect positioning of the intestines. Related defects may occur in other organs, such as the kidney.

Other diseases and conditions related to PKCλ that can be diagnosed, prevented, or treated according to the invention include those that are characterized by abnormalities in tight junctions. As is noted above, we have found that abnormalities in PKCλ (caused, e.g., by the *has* mutation) can lead to defects in epithelial cell-cell interactions. This is due to abnormalities in the formation of tight junctions, which play

critical roles in the sealing of spaces between the individual epithelial or endothelial cells that make up sheets of these cells that line the cavities of the body (e.g., the gastrointestinal tract, blood vessels, the respiratory tract, and the urinary tract), as well as enclose and protect certain organs (e.g., the brain). These sheets of cells function as selective permeability barriers, and alteration of the permeability of these barriers, due to, e.g., a PKCλ defect, can lead to any of a number of diseases or conditions that are well known in the art. For example, increased permeability of the lining of the gastrointestinal tract can lead to Crohn's disease, acute gastroenteritis, and diarrhea. Also, defects in tight junctions can interfere with the critical functions of the blood/brain barrier or the blood/retina barrier. As an additional example, vascular permeability defects in diabetic patients can lead to conditions such as diabetic retinopathy. Additional diseases and conditions that can be diagnosed, prevented, or treated, according to the invention, include those that are associated with abnormalities in epithelial cell polarity, such as polycystic kidney disease (e.g., autosomal dominant polycystic kidney disease). Also, because we have found that abnormalities in PKCλ lead to defects in cell growth control, a role for PKCλ in cancer is indicated. Compounds that are found to modulate PKCλ activity, thus, can be used in the prevention and treatment of cancer, such as, for example, carcinomas (e.g., renal cell carcinoma), which are cancers derived from epithelial cells.

Diagnostic Methods

Nucleic acid molecules encoding PKCλ proteins, as well as polypeptides encoded by these nucleic acid molecules and antibodies specific for these polypeptides, can be used in methods to diagnose or to monitor diseases and conditions involving mutations in, or inappropriate expression of, genes encoding this protein.

The diagnostic methods of the invention can be used, for example, with patients that have a disease or condition associated with PKCλ, in an effort to determine its etiology and, thus, to facilitate selection of an appropriate course of treatment. The diagnostic methods can also be used with patients who have not yet developed, but who are at risk of developing, such a disease or condition, or with patients that are at an early stage of developing such a disease or condition. Also, the diagnostic methods of the invention can be used in prenatal genetic screening, for example, to identify parents who may be carriers of a recessive mutation in a gene encoding a PKCλ protein. The

methods of the invention can be used to diagnose (or to treat) the disorders described herein in any mammal, for example, in humans, domestic pets, or livestock.

Abnormalities in PKCλ that can be detected using the diagnostic methods of the invention include those characterized by, for example, (i) a gene encoding a PKCλ protein containing a mutation that results in the production of an abnormal PKCλ protein, (ii) an abnormal PKCλ polypeptide itself (e.g., a truncated protein), and (iii) a mutation in a PKCλ gene that results in production of an abnormal amount of this protein. Detection of such abnormalities can be used to diagnose human diseases or conditions related to PKCλ , such as those affecting the heart. Exemplary of the mutations in PKCλ genes is the *heart and soul* mutation, which is described further below.

A mutation in a PKCλ gene can be detected in any tissue of a subject, even one in which this protein is not expressed. Because of the possibly limited number of tissues in which these proteins may be expressed, for limited time periods, and because of the possible undesirability of sampling such tissues (e.g., heart tissue) for assays, it may be preferable to detect mutant genes in other, more easily obtained sample types, such as in blood or amniotic fluid samples.

Detection of a mutation in a gene encoding a PKCλ protein can be carried out using any standard diagnostic technique. For example, a biological sample obtained from a patient can be analyzed for one or more mutations (e.g., a *heart and soul* mutation) in nucleic acid molecules encoding a PKCλ protein using a mismatch detection approach. Generally, this approach involves polymerase chain reaction (PCR) amplification of nucleic acid molecules from a patient sample, followed by identification of a mutation (i.e., a mismatch) by detection of altered hybridization, aberrant electrophoretic gel migration, binding, or cleavage mediated by mismatch binding proteins, or by direct nucleic acid molecule sequencing. Any of these techniques can be used to facilitate detection of a mutant gene encoding a PKCλ protein, and each is well known in the art. For instance, examples of these techniques are described by Orita et al. (Proc. Natl. Acad. Sci. U.S.A. 86:2766-2770, 1989) and Sheffield et al. (Proc. Natl. Acad. Sci. U.S.A. 86:232-236, 1989).

As noted above, in addition to facilitating diagnosis of an existing disease or condition, mutation detection assays also provide an opportunity to diagnose a predisposition to disease related to a mutation in a PKCλ gene before the onset of

symptoms. For example, a patient who is heterozygous for a gene encoding an abnormal PKCλ protein (or an abnormal amount thereof) that suppresses normal PKCλ biological activity or expression may show no clinical symptoms of a disease related to such proteins, and yet possess a higher than normal probability of developing such disease. Given such a diagnosis, a patient can take precautions to minimize exposure to adverse environmental factors, and can carefully monitor their medical condition, for example, through frequent physical examinations. As mentioned above, this type of diagnostic approach can also be used to detect a mutation in a gene encoding the PKCλ protein in prenatal screens.

While it may be preferable to carry out diagnostic methods for detecting a mutation in a PKCλ gene using genomic DNA from readily accessible tissues, as noted above, mRNA encoding this protein, or the protein itself, can also be assayed from tissue samples in which it is expressed. Expression levels of a gene encoding PKCλ in such a tissue sample from a patient can be determined by using any of a number of standard techniques that are well known in the art, including northern blot analysis and quantitative PCR (see, e.g., Ausubel et al., supra; PCR Technology: Principles and Applications for DNA Amplification, H.A. Ehrlich, Ed., Stockton Press, NY; Yap et al. Nucl. Acids. Res. 19:4294, 1991).

In another diagnostic approach of the invention, an immunoassay is used to detect or to monitor the level of a PKCλ protein in a biological sample. Polyclonal or monoclonal antibodies specific for the PKCλ protein can be used in any standard immunoassay format (e.g., ELISA, Western blot, or RIA; see, e.g., Ausubel et al., supra) to measure polypeptide the levels of PKCλ. These levels can be compared to levels of PKCλ in a sample from an unaffected individual. Detection of a decrease in production of PKCλ using this method, for example, may be indicative of a condition or a predisposition to a condition involving insufficient biological activity of the PKCλ protein.

Immunohistochemical techniques can also be utilized for detection of PKCλ protein in patient samples. For example, a tissue sample can be obtained from a patient, sectioned, and stained for the presence of PKCλ using an anti-PKCλ antibody and any standard detection system (e.g., one that includes a secondary antibody conjugated to an enzyme, such as horseradish peroxidase). General guidance regarding such techniques

can be found in, e.g., Bancroft et al., Theory and Practice of Histological Techniques, Churchill Livingstone, 1982, and Ausubel et al., supra.

Identification of Molecules that can be used to Treat or to Prevent Diseases or Conditions Associated with PKCλ

Identification of a mutation in the gene encoding PKCλ as resulting in a phenotype that results in abnormal heart growth and development facilitates the identification of molecules (e.g., small organic or inorganic molecules, antibodies, peptides, or nucleic acid molecules) that can be used to treat or to prevent diseases or conditions associated with PKCλ, as discussed above. The effects of candidate compounds on such diseases or conditions can be investigated using, for example, the zebrafish system. As is mentioned above, the zebrafish, *Danio rerio*, is a convenient organism to use in the genetic analysis of development. It has an accessible and transparent embryo, allowing direct observation of organ function from the earliest stages of development, has a short generation time, and is fecund. As discussed further below, zebrafish and other animals having a PKCλ mutation, such as the *heart and soul* mutation, which can be used in these methods, are also included in the invention.

In one example of the screening methods of the invention, a zebrafish having a mutation in a gene encoding the PKCλ protein (e.g., a zebrafish having the *heart and soul* mutation) is contacted with a candidate compound, and the effect of the compound on the development of a heart abnormality, or on the status of such an existing abnormality, is monitored relative to an untreated, identically mutant control. In a variation of this method, a zebrafish, with or without a mutation in the PKCλ gene (e.g., the *has* mutation), is contacted with a candidate compound in the presence of concentramide.

After a compound has been shown to have a desired effect in the zebrafish system, it can be tested in other models of heart disease, for example, in mice or other animals having a mutation in a gene encoding PKCλ. Alternatively, testing in such animal model systems can be carried out in the absence of zebrafish testing. Compounds of the invention can also be tested in animal models of cancer.

Cell culture-based assays can also be used in the identification of molecules that increase or decrease PKCλ levels or biological activity. According to one approach, candidate molecules are added at varying concentrations to the culture medium of cells

expressing PKCλ mRNA. PKCλ biological activity is then measured using standard techniques. The measurement of biological activity can include the measurement of PKCλ protein and nucleic acid molecule levels.

In general, novel drugs for the prevention or treatment of diseases related to mutations in genes encoding PKCλ can be identified from large libraries of natural products, synthetic (or semi-synthetic) extracts, and chemical libraries using methods that are well known in the art. Those skilled in the field of drug discovery and development will understand that the precise source of test extracts or compounds is not critical to the screening methods of the invention and that dereplication, or the elimination of replicates or repeats of materials already known for their therapeutic activities for PKCλ, can be employed whenever possible.

Candidate compounds to be tested include purified (or substantially purified) molecules or one or more components of a mixture of compounds (e.g., an extract or supernatant obtained from cells; Ausubel et al., supra), and such compounds further include both naturally occurring or artificially derived chemicals and modifications of existing compounds. For example, candidate compounds can be polypeptides, synthesized organic or inorganic molecules, naturally occurring organic or inorganic molecules, nucleic acid molecules, and components thereof.

Numerous sources of naturally occurring candidate compounds are readily available to those skilled in the art. For example, naturally occurring compounds can be found in cell (including plant, fungal, prokaryotic, and animal) extracts, mammalian serum, growth medium in which mammalian cells have been cultured, protein expression libraries, or fermentation broths. In addition, libraries of natural compounds in the form of bacterial, fungal, plant, and animal extracts are commercially available from a number of sources, including Biotics (Sussex, UK), Xenova (Slough, UK), Harbor Branch Oceanographic Institute (Ft. Pierce, FL), and PharmaMar, U.S.A. (Cambridge, MA). Furthermore, libraries of natural compounds can be produced, if desired, according to methods that are known in the art, e.g., by standard extraction and fractionation.

Artificially derived candidate compounds are also readily available to those skilled in the art. Numerous methods are available for generating random or directed synthesis (e.g., semi-synthesis or total synthesis) of any number of chemical compounds, including, for example, saccharide-, lipid-, peptide-, and nucleic acid molecule-based

compounds. In addition, synthetic compound libraries are commercially available from Brandon Associates (Merrimack, NH) and Aldrich Chemicals (Milwaukee, WI). Libraries of synthetic compounds can also be produced, if desired, according to methods known in the art, e.g., by standard extraction and fractionation. Furthermore, if desired, any library or compound can be readily modified using standard chemical, physical, or biochemical methods. The techniques of modern synthetic chemistry, including combinatorial chemistry, can also be used (reviewed in Schreiber, Bioorganic and Medicinal Chemistry 6:1172-1152, 1998; Schreiber, Science 287:1964-1969, 2000).

When a crude extract is found to have an effect on the development or persistence of a PKCλ-associated disease, further fractionation of the positive lead extract can be carried out to isolate chemical constituents responsible for the observed effect. Thus, the goal of the extraction, fractionation, and purification process is the careful characterization and identification of a chemical entity within the crude extract having a desired activity. The same assays described herein for the detection of activities in mixtures of compounds can be used to purify the active component and to test derivatives of these compounds. Methods of fractionation and purification of such heterogeneous extracts are well known in the art. If desired, compounds shown to be useful agents for treatment can be chemically modified according to methods known in the art.

In general, compounds that are found to activate PKCλ expression or activity may be used in the prevention or treatment of diseases or conditions of heart, such as those that are characterized by abnormal growth or development, or heart failure (also see above). Compounds that are found to modulate, e.g., block PKCλ expression or activity may be used to prevent or to treat cancer.

Animal Model Systems

The invention also provides animal model systems for use in carrying out the screening methods described above. Examples of these model systems include zebrafish and other animals, such as mice, that have a mutation (e.g., the *heart and soul* mutation) in a PKCλ gene. For example, a zebrafish model that can be used in the invention can include a mutation that results in a lack of PKCλ protein production or production of a truncated (e.g., by introduction of a stop codon) or otherwise altered PKCλ gene product.

As a specific example, a zebrafish having the *heart and soul* mutation can be used (see below).

Treatment or Prevention of PKCλ -Associated Diseases or Conditions

Compounds identified using the screening methods described above can be used to treat patients that have or are at risk of developing diseases or conditions of the heart or cancer. Nucleic acid molecules encoding the PKCλ protein, as well as these proteins themselves, can also be used in such methods. Treatment may be required only for a short period of time or may, in some form, be required throughout a patient's lifetime. Any continued need for treatment, however, can be determined using, for example, the diagnostic methods described above. In considering various therapies, it is to be understood that such therapies are, preferably, targeted to the affected or potentially affected organ (e.g., the heart). Such targeting can be achieved using standard methods.

Treatment or prevention of diseases resulting from a mutated PKCλ gene can be accomplished, for example, by modulating the function of a mutant PKCλ protein. Treatment can also be accomplished by delivering normal PKCλ protein to appropriate cells, altering the levels of normal or mutant PKCλ protein, replacing a mutant gene encoding a PKCλ protein with a normal gene encoding a PKCλ protein, or administering a normal gene encoding a PKCλ protein. It is also possible to correct the effects of a defect in a gene encoding a PKCλ protein by modifying the physiological pathway (e.g., a signal transduction pathway) in which a PKCλ protein participates.

In a patient diagnosed as being heterozygous for a gene encoding a mutant PKCλ protein, or as susceptible to such mutations or aberrant PKCλ expression (even if those mutations or expression patterns do not yet result in alterations in expression or biological activity of PKCλ), any of the therapies described herein can be administered before the occurrence of the disease phenotype. In particular, compounds shown to have an effect on the phenotype of mutants, or to modulate expression of PKCλ proteins, can be administered to patients diagnosed with potential or actual disease by any standard dosage and route of administration.

Any appropriate route of administration can be employed to administer a compound identified as described above, a PKCλ gene, or a PKCλ protein, according to the invention. For example, administration can be parenteral, intravenous, intra-arterial,

subcutaneous, intramuscular, intraventricular, intracapsular, intraspinal, intracisternal, intraperitoneal, intranasal, by aerosol, by suppository, or oral.

A therapeutic compound of the invention can be administered within a pharmaceutically acceptable diluent, carrier, or excipient, in unit dosage form. Administration can begin before or after the patient is symptomatic. Methods that are well known in the art for making formulations are found, for example, in Remington's Pharmaceutical Sciences (18^{th} edition), ed. A. Gennaro, 1990, Mack Publishing Company, Easton, PA. Therapeutic formulations can be in the form of liquid solutions or suspensions. Formulations for parenteral administration can contain, for example, excipients, sterile water, or saline; polyalkylene glycols, such as polyethylene glycol; oils of vegetable origin; or hydrogenated napthalenes. Biocompatible, biodegradable lactide polymer, lactide/glycolide copolymer, or polyoxyethylene-polyoxypropylene copolymers can be used to control the release of the compounds. Other potentially useful parenteral delivery systems include ethylene-vinyl acetate copolymer particles, osmotic pumps, implantable infusion systems, and liposomes. For oral administration, formulations can be in the form of tablets or capsules. Formulations for inhalation can contain excipients, for example, lactose, or can be aqueous solutions containing, for example, polyoxyethylene-9-lauryl ether, glycocholate, and deoxycholate, or can be oily solutions for administration in the form of nasal drops or as a gel. Alternatively, intranasal formulations can be in the form of powders or aerosols.

To replace a mutant protein with normal protein, or to add protein to cells that do not express a sufficient amount of PKCλ or normal PKCλ, it may be necessary to obtain large amounts of pure PKCλ protein from cell culture systems in which the protein is expressed (see, e.g., below). Delivery of the protein to the affected tissue can then be accomplished using appropriate packaging or administration systems.

Gene therapy is another therapeutic approach for preventing or ameliorating diseases caused by PKCλ gene defects. Nucleic acid molecules encoding wild type PKCλ protein can be delivered to cells that lack sufficient, normal PKCλ protein biological activity (e.g., cells carrying mutations (e.g., the *heart and soul* mutation) in PKCλ genes). The nucleic acid molecules must be delivered to those cells in a form in which they can be taken up by the cells and so that sufficient levels of protein, to provide effective PKCλ protein function, can be produced. Alternatively, for some PKCλ mutations, it may be possible to slow the progression of the resulting disease or to

modulate PKCλ protein activity by introducing another copy of a homologous gene bearing a second mutation in that gene, to alter the mutation, or to use another gene to block any negative effect.

Transducing viral (e.g., retroviral, adenoviral, and adeno-associated viral) vectors can be used for somatic cell gene therapy, especially because of their high efficiency of infection and stable integration and expression (see, e.g., Cayouette et al., Human Gene Therapy 8:423-430, 1997; Kido et al., Current Eye Research 15:833-844, 1996; Bloomer et al., Journal of Virology 71:6641-6649, 1997; Naldini et al., Science 272:263-267, 1996; and Miyoshi et al., Proc. Natl. Acad. Sci. U.S.A. 94:10319, 1997). For example, the full length PKCλ gene, or a portion thereof, can be cloned into a retroviral vector and expression can be driven from its endogenous promoter, from the retroviral long terminal repeat, or from a promoter specific for a target cell type of interest. Other viral vectors that can be used include, for example, a vaccinia virus, a bovine papilloma virus, or a herpes virus, such as Epstein-Barr Virus (also see, for example, the vectors of Miller, Human Gene Therapy 15-14, 1990; Friedman, Science 244:1275-1281, 1989; Eglitis et al., BioTechniques 6:608-614, 1988; Tolstoshev et al., Current Opinion in Biotechnology 1:55-61, 1990; Sharp, The Lancet 337:1277-1278, 1991; Cornetta et al., Nucleic Acid Research and Molecular Biology 36:311-322, 1987; Anderson, Science 226:401-409, 1984; Moen, Blood Cells 17:407-416, 1991; Miller et al., Biotechnology 7:980-990, 1989; Le Gal La Salle et al., Science 259:988-990, 1993; and Johnson, Chest 107:77S-83S, 1995). Retroviral vectors are particularly well developed and have been used in clinical settings (Rosenberg et al., N. Engl. J. Med 323:370, 1990; Anderson et al., U.S. Patent No. 5,399,346).

Non-viral approaches can also be employed for the introduction of therapeutic DNA into cells predicted to be subject to diseases involving the PKCλ protein. For example, a PKCλ nucleic acid molecule or an antisense nucleic acid molecule can be introduced into a cell by lipofection (Felgner et al., Proc. Natl. Acad. Sci. U.S.A. 84:7413, 1987; Ono et al., Neuroscience Letters 17:259, 1990; Brigham et al., Am. J. Med. Sci. 298:278, 1989; Staubinger et al., Methods in Enzymology 101:512, 1983), asialoorosomucoid-polylysine conjugation (Wu et al., Journal of Biological Chemistry 263:14621, 1988; Wu et al., Journal of Biological Chemistry 264:16985, 1989), or by micro-injection under surgical conditions (Wolff et al., Science 247:1465, 1990).

Gene transfer can also be achieved using non-viral means involving transfection *in vitro*. Such methods include the use of calcium phosphate, DEAE dextran, electroporation, and protoplast fusion. Liposomes can also be potentially beneficial for delivery of DNA into a cell. Transplantation of normal genes into the affected tissues of a patient can also be accomplished by transferring a normal PKCλ protein into a cultivatable cell type *ex vivo* (e.g., an autologous or heterologous primary cell or progeny thereof), after which the cell (or its descendants) are injected into a targeted tissue.

PKCλ cDNA expression for use in gene therapy methods can be directed from any suitable promoter (e.g., the human cytomegalovirus (CMV), simian virus 40 (SV40), or metallothionein promoters), and regulated by any appropriate mammalian regulatory element. For example, if desired, enhancers known to preferentially direct gene expression in specific cell types can be used to direct PKCλ expression. The enhancers used can include, without limitation, those that are characterized as tissue- or cell-specific enhancers. Alternatively, if a PKCλ genomic clone is used as a therapeutic construct (such clones can be identified by hybridization with PKCλ cDNA, as described herein), regulation can be mediated by the cognate regulatory sequences or, if desired, by regulatory sequences derived from a heterologous source, including any of the promoters or regulatory elements described above.

Molecules for effecting antisense-based strategies can be employed to explore PKCλ protein gene function, as a basis for therapeutic drug design, as well as to treat PKCλ-associated diseases, such as cancer. These strategies are based on the principle that sequence-specific suppression of gene expression (via transcription or translation) can be achieved by intracellular hybridization between genomic DNA or mRNA and a complementary antisense species. The formation of a hybrid RNA duplex interferes with transcription of the target PKCλ-encoding genomic DNA molecule, or processing, transport, translation, or stability of the target PKCλ mRNA molecule.

Antisense strategies can be delivered by a variety of approaches. For example, antisense oligonucleotides or antisense RNA can be directly administered (e.g., by intravenous injection) to a subject in a form that allows uptake into cells. Alternatively, viral or plasmid vectors that encode antisense RNA (or antisense RNA fragments) can be introduced into a cell *in vivo* or *ex vivo*. Antisense effects can be induced by control (sense) sequences; however, the extent of phenotypic changes is highly variable.

Phenotypic effects induced by antisense molecules are based on changes in criteria such as protein levels, protein activity measurement, and target mRNA levels.

PKCλ gene therapy can also be accomplished by direct administration of antisense PKCλ mRNA to a cell that is expected to be adversely affected by the expression of wild type or mutant PKCλ protein. The antisense PKCλ mRNA can be produced and isolated by any standard technique, but is most readily produced by *in vitro* transcription using an antisense PKCλ cDNA under the control of a high efficiency promoter (e.g., the T7 promoter). Administration of antisense PKCλ mRNA to cells can be carried out by any of the methods for direct nucleic acid molecule administration described above.

An alternative strategy for inhibiting PKCλ protein function using gene therapy involves intracellular expression of an anti-PKCλ protein antibody or a portion of an anti-PKCλ protein antibody. For example, the gene (or gene fragment) encoding a monoclonal antibody that specifically binds to a PKCλ protein and inhibits its biological activity can be placed under the transcriptional control of a tissue-specific gene regulatory sequence.

Another therapeutic approach included in the invention involves administration of a recombinant PKCλ polypeptide, either directly to the site of a potential or actual disease-affected tissue (for example, by injection) or systemically (for example, by any conventional recombinant protein administration technique). The dosage of the PKCλ protein depends on a number of factors, including the size and health of the individual patient but, generally, between 0.1 mg and 100 mg, inclusive, is administered per day to an adult in any pharmaceutically acceptable formulation.

In addition to the therapeutic methods described herein, involving administration of PKCλ-modulating compounds, PKCλ proteins, or PKCλ nucleic acids to patients, the invention provides methods of culturing organs in the presence of such molecules. In particular, as is noted above, a PKCλ mutation is associated with abnormal heart growth and development. Thus, culturing heart tissue in the presence of these molecules can be used to promote its growth and development. This tissue can be that which is being prepared for transplant from, e.g., an allogeneic or xenogeneic donor, as well as synthetic tissue or organs.

Synthesis of PKCλ Proteins, Polypeptides, and Polypeptide Fragments

Those skilled in the art of molecular biology will understand that a wide variety of expression systems can be used to produce recombinant PKCλ proteins. As discussed further below, the precise host cell used is not critical to the invention. The PKCλ proteins can be produced in a prokaryotic host (e.g., *E. coli*) or in a eukaryotic host (e.g., *S. cerevisiae*, insect cells, such as Sf9 cells, or mammalian cells, such as COS-1, NIH 3T3, or HeLa cells). These cells are commercially available from, for example, the American Type Culture Collection, Manassas, VA (see also Ausubel et al., supra). The method of transformation and the choice of expression vehicle (e.g., expression vector) will depend on the host system selected. Transformation and transfection methods are described, e.g., in Ausubel et al., supra, and expression vehicles can be chosen from those provided, e.g., in Pouwels et al., Cloning Vectors: A Laboratory Manual, 1985, Supp. 1987. Specific examples of expression systems that can be used in the invention are described further as follows.

For protein expression, eukaryotic or prokaryotic expression systems can be generated in which PKCλ gene sequences are introduced into a plasmid or other vector, which is then used to transform living cells. Constructs in which full-length PKCλ cDNAs, containing the entire open reading frame, inserted in the correct orientation into an expression plasmid, can be used for protein expression. Alternatively, portions of PKCλ gene sequences, including wild type or mutant PKCλ sequences, can be inserted. Prokaryotic and eukaryotic expression systems allow various important functional domains of PKCλ proteins to be recovered, if desired, as fusion proteins, and then used for binding, structural, and functional studies, and also for the generation of antibodies.

Typical expression vectors contain promoters that direct synthesis of large amounts of mRNA corresponding to a nucleic acid molecule that has been inserted into the vector. They can also include a eukaryotic or prokaryotic origin of replication, allowing for autonomous replication within a host cell, sequences that confer resistance to an otherwise toxic drug, thus allowing vector-containing cells to be selected in the presence of the drug, and sequences that increase the efficiency with which the synthesized mRNA is translated. Stable, long-term vectors can be maintained as freely replicating entities by using regulatory elements of, for example, viruses (e.g., the OriP sequences from the Epstein Barr Virus genome). Cell lines can also be produced that

have the vector integrated into genomic DNA of the cells and, in this manner, the gene product can be produced in the cells on a continuous basis.

Expression of foreign molecules in bacteria, such as *Escherichia coli*, requires the insertion of a foreign nucleic acid molecule, e.g., a PKCλ nucleic acid molecule, into a bacterial expression vector. Such plasmid vectors include several elements required for the propagation of the plasmid in bacteria, and for expression of foreign DNA contained within the plasmid. Propagation of only plasmid-bearing bacteria is achieved by introducing, into the plasmid, a selectable marker-encoding gene that allows plasmid-bearing bacteria to grow in the presence of an otherwise toxic drug. The plasmid also contains a transcriptional promoter capable of directing synthesis of large amounts of mRNA from the foreign DNA. Such promoters can be, but are not necessarily, inducible promoters that initiate transcription upon induction by culture under appropriate conditions (e.g., in the presence of a drug that activates the promoter). The plasmid also, preferably, contains a polylinker to simplify insertion of the gene in the correct orientation within the vector.

Once an appropriate expression vector containing a PKCλ gene, or a fragment, fusion, or mutant thereof, is constructed, it can be introduced into an appropriate host cell using a transformation technique, such as, for example, calcium phosphate transfection, DEAE-dextran transfection, electroporation, microinjection, protoplast fusion, or liposome-mediated transfection. Host cells that can be transfected with the vectors of the invention can include, but are not limited to, *E. coli* or other bacteria, yeast, fungi, insect cells (using, for example, baculoviral vectors for expression), or cells derived from mice, humans, or other animals. Mammalian cells can also be used to express PKCλ proteins using a virus expression system (e.g., a vaccinia virus expression system) described, for example, in Ausubel et al., supra.

In vitro expression of PKCλ proteins, fusions, polypeptide fragments, or mutants encoded by cloned DNA can also be carried out using the T7 late-promoter expression system. This system depends on the regulated expression of T7 RNA polymerase, an enzyme encoded in the DNA of bacteriophage T7. The T7 RNA polymerase initiates transcription at a specific 23 base pair promoter sequence called the T7 late promoter. Copies of the T7 late promoter are located at several sites on the T7 genome, but none are present in *E. coli* chromosomal DNA. As a result, in T7-infected *E. coli*, T7 RNA polymerase catalyzes transcription of viral genes, but not *E. coli* genes. In this

expression system, recombinant *E. coli* cells are first engineered to carry the gene encoding T7 RNA polymerase next to the *lac* promoter. In the presence of IPTG, these cells transcribe the T7 polymerase gene at a high rate and synthesize abundant amounts of T7 RNA polymerase. These cells are then transformed with plasmid vectors that carry a copy of the T7 late promoter protein. When IPTG is added to the culture medium containing these transformed *E. coli* cells, large amounts of T7 RNA polymerase are produced. The polymerase then binds to the T7 late promoter on the plasmid expression vectors, catalyzing transcription of the inserted cDNA at a high rate. Since each *E. coli* cell contains many copies of the expression vector, large amounts of mRNA corresponding to the cloned cDNA can be produced in this system and the resulting protein can be radioactively labeled.

Plasmid vectors containing late promoters and the corresponding RNA polymerases from related bacteriophages, such as T3, T5, and SP6, can also be used for *in vitro* production of proteins from cloned DNA. *E. coli* can also be used for expression using an M13 phage, such as mGPI-2. Furthermore, vectors that contain phage lambda regulatory sequences, or vectors that direct the expression of fusion proteins, for example, a maltose-binding protein fusion protein or a glutathione-S-transferase fusion protein, also can be used for expression in *E. coli*.

Eukaryotic expression systems are useful for obtaining appropriate post-translational modification of expressed proteins. Transient transfection of a eukaryotic expression plasmid containing a PKCλ gene into a eukaryotic host cell allows the transient production of a PKCλ protein by the transfected host cell. PKCλ proteins can also be produced by a stably-transfected eukaryotic (e.g., mammalian) cell line. A number of vectors suitable for stable transfection of mammalian cells are available to the public (see, e.g., Pouwels et al., supra), as are methods for constructing lines including such cells (see, e.g., Ausubel et al., supra).

In one example, cDNA encoding a PKCλ protein, fusion, mutant, or polypeptide fragment is cloned into an expression vector that includes the dihydrofolate reductase (DHFR) gene. Integration of the plasmid and, therefore, integration of the *heart and soul* protein-encoding gene, into the host cell chromosome is selected for by inclusion of 0.01-300 μM methotrexate in the cell culture medium (Ausubel et al., supra). This dominant selection can be accomplished in most cell types. Recombinant protein expression can be increased by DHFR-mediated amplification of the transfected gene.

Methods for selecting cell lines bearing gene amplifications are described in Ausubel et al., supra. These methods generally involve extended culture in medium containing gradually increasing levels of methotrexate. The most commonly used DHFR-containing expression vectors are pCVSEII-DHFR and pAdD26SV(A) (described, for example, in Ausubel et al., supra). The host cells described above or, preferably, a DHFR-deficient CHO cell line (e.g., CHO DHFR- cells, ATCC Accession No. CRL 9096) are among those that are most preferred for DHFR selection of a stably transfected cell line or DHFR-mediated gene amplification.

Another preferred eukaryotic expression system is the baculovirus system using, for example, the vector pBacPAK9, which is available from Clontech (Palo Alto, CA). If desired, this system can be used in conjunction with other protein expression techniques, for example, the myc tag approach described by Evan et al. (Molecular and Cellular Biology 5:3610-3616, 1985).

Once a recombinant protein is expressed, it can be isolated from the expressing cells by cell lysis followed by protein purification techniques, such as affinity chromatography. In this example, an anti-PKCλ antibody, which can be produced by the methods described herein, can be attached to a column and used to isolate the recombinant PKCλ. Lysis and fractionation of PKCλ-harboring cells prior to affinity chromatography can be performed by standard methods (see, e.g., Ausubel et al., supra). Once isolated, the recombinant protein can, if desired, be purified further by, e.g., high performance liquid chromatography (HPLC; e.g., see Fisher, *Laboratory Techniques In Biochemistry and Molecular Biology*, Work and Burdon, Eds., Elsevier, 1980).

Polypeptides of the invention, particularly short PKCλ fragments and longer fragments of the N-terminus and C-terminus of PKCλ, can also be produced by chemical synthesis (e.g., by the methods described in *Solid Phase Peptide Synthesis*, 2nd ed., 1984, The Pierce Chemical Co., Rockford, IL). These general techniques of polypeptide expression and purification can also be used to produce and isolate useful PKCλ fragments or analogs, as described herein.

PKCλ Protein Fragments

Polypeptide fragments that include various portions of PKCλ proteins are useful in identifying the domains of PKCλ that are important for its biological activities. Methods for generating such fragments are well known in the art (see, for example,

Ausubel et al., supra), using the nucleotide sequences provided herein. For example, a PKCλ protein fragment can be generated by PCR amplifying a desired PKCλ nucleic acid molecule fragment using oligonucleotide primers designed based upon PKCλ nucleic acid sequences. Preferably, the oligonucleotide primers include unique restriction enzyme sites that facilitate insertion of the amplified fragment into the cloning site of an expression vector (e.g., a mammalian expression vector, see above). This vector can then be introduced into a cell (e.g., a mammalian cell; see above) by artifice, using any of the various techniques that are known in the art, such as those described herein, resulting in the production of a PKCλ protein fragment in the cell containing the expression vector. PKCλ protein fragments (e.g., chimeric fusion proteins) can also be used to raise antibodies specific for various regions of the PKCλ protein using, for example, the methods described below.

PKCλ Protein Antibodies

To prepare polyclonal antibodies, PKCλ proteins, fragments of PKCλ proteins, or fusion proteins containing defined portions of PKCλ proteins can be synthesized in, e.g., bacteria by expression of corresponding DNA sequences contained in a suitable cloning vehicle. Fusion proteins are commonly used as a source of antigen for producing antibodies. Two widely used expression systems for *E. coli* are *lacZ* fusions using the pUR series of vectors and *trpE* fusions using the pATH vectors. The proteins can be purified, coupled to a carrier protein, mixed with Freund's adjuvant to enhance stimulation of the antigenic response in an inoculated animal, and injected into rabbits or other laboratory animals. Alternatively, protein can be isolated from PKCλ-expressing cultured cells. Following booster injections at bi-weekly intervals, the rabbits or other laboratory animals are then bled and the sera isolated. The sera can be used directly or can be purified prior to use by various methods, including affinity chromatography employing reagents such as Protein A-Sepharose, antigen-Sepharose, and anti-mouse-Ig-Sepharose. The sera can then be used to probe protein extracts from PKCλ-expressing tissue fractionated by polyacrylamide gel electrophoresis to identify PKCλ proteins. Alternatively, synthetic peptides can be made that correspond to antigenic portions of the protein and used to inoculate the animals.

To generate peptide or full-length protein for use in making, for example, PKCλ-specific antibodies, a PKCλ coding sequence can be expressed as a C-terminal or N-terminal fusion with glutathione S-transferase (GST; Smith et al., Gene 67:31-40, 1988). The fusion protein can be purified on glutathione-Sepharose beads, eluted with glutathione, cleaved with a protease, such as thrombin or Factor-Xa (at the engineered cleavage site), and purified to the degree required to successfully immunize rabbits. Primary immunizations can be carried out with Freund's complete adjuvant and subsequent immunizations performed with Freund's incomplete adjuvant. Antibody titers can be monitored by Western blot and immunoprecipitation analyses using the protease-cleaved PKCλ fragment of the GST-PKCλ protein. Immune sera can be affinity purified using CNBr-Sepharose-coupled PKCλ. Antiserum specificity can be determined using a panel of unrelated GST fusion proteins.

Alternatively, monoclonal PKCλ antibodies can be produced by using, as an antigen, PKCλ isolated from PKCλ-expressing cultured cells or PKCλ protein isolated from tissues. The cell extracts, or recombinant protein extracts containing PKCλ, can, for example, be injected with Freund's adjuvant into mice. Several days after being injected, the mouse spleens can be removed, the tissues disaggregated, and the spleen cells suspended in phosphate buffered saline (PBS). The spleen cells serve as a source of lymphocytes, some of which would be producing antibody of the appropriate specificity. These can then be fused with permanently growing myeloma partner cells, and the products of the fusion plated into a number of tissue culture wells in the presence of selective agents, such as hypoxanthine, aminopterine, and thymidine (HAT). The wells can then be screened by ELISA to identify those containing cells making antibodies capable of binding to PKCλ, polypeptide fragment, or mutant thereof. These cells can then be re-plated and, after a period of growth, the wells containing these cells can be screened again to identify antibody-producing cells. Several cloning procedures can be carried out until over 90% of the wells contain single clones that are positive for specific antibody production. From this procedure, a stable line of clones that produce the antibody can be established. The monoclonal antibody can then be purified by affinity chromatography using Protein A Sepharose and ion exchange chromatography, as well as variations and combinations of these techniques. Once produced, monoclonal antibodies are also tested for specific PKCλ recognition by Western blot or immunoprecipitation analysis (see, e.g., Kohler et al., Nature 256:495, 1975; Kohler et

al., European Journal of Immunology 6:511, 1976; Kohler et al., European Journal of Immunology 6:292, 1976; Hammerling et al., In Monoclonal Antibodies and T Cell Hybridomas, Elsevier, New York, NY, 1981; Ausubel et al., supra).

As an alternate or adjunct immunogen to GST fusion proteins, peptides corresponding to relatively unique hydrophilic regions of PKCλ can be generated and coupled to keyhole limpet hemocyanin (KLH) through an introduced C-terminal lysine. Antiserum to each of these peptides can be similarly affinity-purified on peptides conjugated to BSA, and specificity tested by ELISA and Western blotting using peptide conjugates, and by Western blotting and immunoprecipitation using PKCλ, for example, expressed as a GST fusion protein.

Antibodies of the invention can be produced using PKCλ amino acid sequences that do not reside within highly conserved regions, and that appear likely to be antigenic, as analyzed by criteria such as those provided by the Peptide Structure Program (Genetics Computer Group Sequence Analysis Package, Program Manual for the GCG Package, Version 7, 1991) using the algorithm of Jameson et al., CABIOS 4:181, 1988. These fragments can be generated by standard techniques, e.g., by PCR, and cloned into the pGEX expression vector. GST fusion proteins can be expressed in *E. coli* and purified using a glutathione-agarose affinity matrix (Ausubel et al., supra). To generate rabbit polyclonal antibodies, and to minimize the potential for obtaining antisera that is non-specific, or exhibits low-affinity binding to PKCλ, two or three fusions are generated for each protein, and each fusion is injected into at least two rabbits. Antisera are raised by injections in series, preferably including at least three booster injections.

In addition to intact monoclonal and polyclonal anti-PKCλ antibodies, the invention features various genetically engineered antibodies, humanized antibodies, and antibody fragments, including F(ab')2, Fab', Fab, Fv, and sFv fragments. Truncated versions of monoclonal antibodies, for example, can be produced by recombinant methods in which plasmids are generated that express the desired monoclonal antibody fragment(s) in a suitable host. Antibodies can be humanized by methods known in the art, e.g., monoclonal antibodies with a desired binding specificity can be commercially humanized (Scotgene, Scotland; Oxford Molecular, Palo Alto, CA). Fully human antibodies, such as those expressed in transgenic animals, are also included in the invention (Green et al., Nature Genetics 7:13-21, 1994).

Ladner (U.S. Patent Nos. 4,946,778 and 4,704,692) describes methods for preparing single polypeptide chain antibodies. Ward et al., Nature 341:544-546, 1989, describes the preparation of heavy chain variable domains, which they term "single domain antibodies," and which have high antigen-binding affinities. McCafferty et al., Nature 348:552-554, 1990, shows that complete antibody V domains can be displayed on the surface of fd bacteriophage, that the phage bind specifically to antigen, and that rare phage (one in a million) can be isolated after affinity chromatography. Boss et al., U.S. Patent No. 4,816,397, describes various methods for producing immunoglobulins, and immunologically functional fragments thereof, that include at least the variable domains of the heavy and light chains in a single host cell. Cabilly et al., U.S. Patent No. 4,816,567, describes methods for preparing chimeric antibodies.

Use of PKCλ Antibodies

Antibodies to PKCλ can be used, as noted above, to detect PKCλ or to inhibit the biological activities of PKCλ. For example, a nucleic acid molecule encoding an antibody or portion of an antibody can be expressed within a cell to inhibit PKCλ function. In addition, the antibodies can be coupled to compounds, such as radionuclides and liposomes, for diagnostic or therapeutic uses. Antibodies that inhibit the activity of a PKCλ polypeptide described herein can also be useful in preventing or slowing the development of a disease caused by inappropriate expression of a wild type or mutant PKCλ gene.

Detection of PKCλ Gene Expression

As noted, the antibodies described above can be used to monitor PKCλ gene expression. *In situ* hybridization of RNA can be used to detect the expression of PKCλ genes. RNA *in situ* hybridization techniques rely upon the hybridization of a specifically labeled nucleic acid probe to the cellular RNA in individual cells or tissues. Therefore, RNA *in situ* hybridization is a powerful approach for studying tissue- and temporal-specific gene expression. In this method, oligonucleotides, cloned DNA fragments, or antisense RNA transcripts of cloned DNA fragments corresponding to unique portions of PKCλ genes are used to detect specific mRNA species, e.g., in the tissues of animals, such as mice, at various developmental stages. Other gene expression detection

techniques are known to those of skill in the art and can be employed for detection of PKCλ gene expression.

Identification of Additional PKCλ Genes

Standard techniques, such as the polymerase chain reaction (PCR) and DNA hybridization, can be used to clone PKCλ gene homologues in other species and PKCλ-related genes in humans. PKCλ-related genes and homologues can be readily identified using low-stringency DNA hybridization or low-stringency PCR with human PKCλ probes or primers. Degenerate primers encoding human PKCλ or human PKCλ-related amino acid sequences can be used to clone additional PKCλ-related genes and homologues by RT-PCR.

Construction of Transgenic Animals and Knockout Animals

Characterization of PKCλ genes provides information that allows PKCλ knockout animal models to be developed by homologous recombination. Preferably, a PKCλ knockout animal is a mammal, most preferably a mouse. Similarly, animal models of PKCλ overproduction can be generated by integrating one or more PKCλ sequences into the genome of an animal, according to standard transgenic techniques. Moreover, the effect of PKCλ mutations (e.g., dominant gene mutations) can be studied using transgenic mice carrying mutated PKCλ transgenes or by introducing such mutations into the endogenous PKCλ gene, using standard homologous recombination techniques.

A replacement-type targeting vector, which can be used to create a knockout model, can be constructed using an isogenic genomic clone, for example, from a mouse strain such as 129/Sv (Stratagene Inc., LaJolla, CA). The targeting vector can be introduced into a suitably derived line of embryonic stem (ES) cells by electroporation to generate ES cell lines that carry a profoundly truncated form of a PKCλ gene. To generate chimeric founder mice, the targeted cell lines are injected into a mouse blastula-stage embryo. Heterozygous offspring can be interbred to homozygosity. PKCλ knockout mice provide a tool for studying the role of PKCλ in embryonic development and in disease. Moreover, such mice provide the means, *in vivo*, for testing therapeutic compounds for amelioration of diseases or conditions involving PKCλ-dependent or a PKCλ-effected pathway.

Use of PKCλ as a Marker for Stem Cells of the Heart

As PKCλ is expressed in cells that give rise to the heart during the course of development, it can be used as a marker for stem cells of the heart. For example, PKCλ can be used to identify, sort, or target such stem cells. A pool of candidate cells, for example, can be analyzed for PKCλ expression, to facilitate the identification of heart stem cells, which, based on this identification can be separated from the pool. The isolated stem cells can be used for many purposes that are known to those of skill in this art. For example, the stem cells can be used in the production of new organs, in organ culture, or to fortify damaged or transplanted organs.

Experimental Results

Concentramide specifically modulates a biological pathway involved in heart patterning

Zebrafish embryos have recently been shown to be amenable to high-throughput screening to identify small molecules that perturb developmental processes (Peterson et al., Proc. Natl. Acad. Sci. U.S.A. 97:12965-12969, 2000). In one such screen, we exposed developing zebrafish embryos to small molecules from a large, diverse chemical library. Visual inspection of the transparent embryos was used to identify small molecules that affect the global patterning of the heart. One of these small molecules is a biaryl compound containing an acrylamide moiety that we call concentramide (Fig. 1A), originally identified as library number 32P6 (Peterson et al., supra).

Normally, by 24 hours post-fertilization (hpf) the heart tube assembles in the midline, with the atrium anterior to the ventricle and slightly displaced towards the left (Fig. 2A), and blood flow is driven from atrial to ventricular end, first by persistalsis and then by sequential chamber contractions. By 30 hpf, the chambers are clearly demarcated (Fig. 2B, using cardiac myosin light chain 2, cmlc2, to label both chambers) and express different genes, as shown in Fig. 2C (ventricle-specific myosin heavy chain and atrial-specific antibody S46).

Embryos exposed to concentramide develop compact hearts that do not sustain a circulation. It appears that both the atrium and ventricle form and beat in a coordinated manner in these fish, but that the ventricle forms in the center of the atrium, as shown in Figs. 2E and 2F. The result is a heart in which the atrium and ventricle form two concentric rings, the inner ring composed of the ventricle and the outer ring composed of

the atrium. From the dorsal view, the heart looks like a bullseye (Fig. 2D), and from the lateral view, it looks like an inverted mushroom, in which the ventricle forms the stalk of the mushroom and the atrium surrounds and covers the ventricle like a mushroom cap (Fig. 1B).

Several observations suggest that concentramide is a highly specific modulator of a particular molecular pathway critical to heart patterning. Concentramide is very potent, with an ED_{50} of about 2 nM. More importantly, higher doses of concentramide do not appear to cause additional side effects. Concentramide causes virtually the same phenotype when used at a concentration of 6 μM as it does when used at a concentration of 6 nM, suggesting that it modulates a specific molecular target at least 1,000 times more potently than it modulates other proteins affecting visible developmental processes. The effect of concentramide on cardiovascular development does not appear to be a result of general cytotoxicity. Development of concentramide-treated embryos is not delayed relative to untreated siblings, and no increase in cell death is apparent. Concentramide also has no effect on the rate of proliferation of yeast or bromodeoxyuridine incorporation in mammalian cells. Given the potency of concentramide, its phenotypic reproducibility over a broad concentration range, and the rarity of the phenotype it produces (none of the >2000 other small molecules screened generates a similar phenotype), we conclude that concentramide is a specific modulator of a biological pathway responsible for heart patterning.

A time window for concentramide effects

One advantage of small molecules over genetic mutations in studying a developmental process is that small molecules allow the process to be modulated with much greater temporal control. Small molecules can be added or washed away at any time during development, whereas genetic mutations are generally present throughout development. This temporal control afforded by small molecules facilitates the identification of critical periods for developmental processes.

To identify the developmental stage at which concentramide disrupts heart patterning decisions, we added concentramide to the water of developing embryos at various times. As shown in Fig. 1C, embryos treated at any time prior to 14 hpf exhibit the concentric chamber morphology at 24 hpf, while embryos treated after 17 hpf exhibit wild-type heart morphology at 24 hpf. Repeating the experiment with more precise

staging revealed that concentramide must be present before the 14-somite stage (approximately 15 hpf) to induce the concentric chamber morphology. Therefore, a developmental event occurring at the 14-somite stage is critical for heart patterning and is disrupted by the small molecule concentramide.

The hearts of concentramide-treated embryos phenocopy heart-and-soul mutants

Heart-and-soul (*has*) is a mutation isolated in our large-scale genetic screen. The hearts of homozygous *has* mutant embryos are small. We find here that, like those of concentramide-treated embryos, the hearts of *has* mutant embryos have ventricular tissue within the atrium (Figs. 2G and 2H). They manifest radial sequential contractions of the atrium, then the ventricle. The *has* mutant embryos, however, also manifest defects in many tissues including the retina, kidney, gut, and brain. These defects are not present in concentramide-treated embryos. The brains of concentramide-treated embryos develop abnormally, but treating embryos between 9 and 14 hpf eliminates this brain defect, while preserving the concentric heart chamber phenotype (Fig. 1C). Therefore, the heart phenotypes of concentramide-treated and *has* mutant embryos are very similar, but concentramide-treated embryos appear to have fewer developmental defects elsewhere, and the cardiac specificity of the phenotype can be increased further by controlling the timing of concentramide treatment.

Heart-and-soul encodes an atypical PKCλ

Given the phenotypic similarities between hearts from *has* and concentramide-treated embryos, we reasoned that cloning the *has* gene might provide molecular insight about the process of heart patterning. Furthermore, cloning of *has* might allow us to determine whether *has* and concentramide influence heart patterning through similar or distinct mechanisms. We mapped *has* by linkage analysis with zebrafish SSR markers (Michelmore et al., Proc. Natl. Acad. Sci. U.S.A. 88:9828-9832, 1991; Knapik et al., Nat. Genet. 18:338-343, 1998; Shimoda et al., Genomics 58:219-232, 1999) and AFLP (Vos et al., Nucleic Acids Res. 23:4407-4414, 1995) to an interval flanked by markers z8451 and z11023 of approximately 1.1cM (Fig. 3A). These were used to initiate a walk using YACs and BACs, which proceeded by end-cloning, refined mapping, and ultimately sequencing. Genes identified as candidates for the mutation were assayed by *in situ* analysis and for cDNA polymorphism by RT-PCR of wild-type and mutant RNA

pools. The genes contained within the BACs are shown in Table 1. The gene assignments are based on BLASTX alignments.

Table 1. Candidate genes identified within the *heart-and-soul* interval

BAC address	identified genes (GenBank accession#)
109f10/122n17	KIAA0670 protein/acinus (NP_055792)
	membane-type 1 metalloproteinase precursor (AAD13803)
	adaptin, gamma (NP_001119)
	KIAA1416 protein, novel Helicase C-terminal domain and SNF2 N-termina
	domains containing protein, similar to KIAA0308 (CAB57836)
	ZPC domain containing protein 2 (AAD38907)
	zinc finger protein sal (AAB51127)
	cerebellin 1 precursor (NP_004343)
	RING finger protein (AAB05873)
152p21	unknown (NP_056541)
89i15	precerebellin-like protein (AAF04305)
23c14	PKCλ
	transforming protein sno-N - chicken (I51298)
53c17	no genes detected by BLASTX (mostly repetitive)

By sequencing PKCλ from wild type and mutant embryos, we confirmed that both *has* alleles harbor mutations in the PKCλ coding sequence. The mutation in the m567 allele causes a premature stop codon after amino acid 518, and the mutation in the m129 allele causes a premature stop codon after amino acid 514 (Fig. 3A). We determined the complete genomic structure of the zebrafish PKCλ gene by shotgun sequencing of BAC 23c14. It is comprised of 18 exons spanning approximately 45 kb. We find PKCλ mRNA to be expressed in a broad range of tissues.

The C-terminal truncation of PKCλ does not appear to destabilize the protein, as truncated protein is detected by western blot analysis of mutant embryos (Fig. 3B). However, truncation might be predicted to eliminate a domain essential for PKCλ function, given that C-terminal truncation of PKCα or PKCβ renders these related kinases catalytically inactive (Riedel et al., J. Cell. Biochem. 52:320-329, 1993; Riedel et al., Mol. Cell. Biol. 13:4728-4735, 1993). In order to confirm the role of PKCλ mutation in the phenotype, we injected antisense morpholino oligomers complementary to the PKCλ translational start site. These injections phenocopy the mutation entirely. The injected embryos are indistinguishable at the gross morphological level from the

genetic mutants (Fig. 3C), supporting the idea that loss of the C-terminal 70 amino acids is sufficient to eliminate gene function.

The integrity of epithelia is affected by PKCλ mutation, but not by treatment with concentramide

PKCλ belongs to the large PKC family of kinases and, with PKCζ, is classified as an 'atypical' PKC (Mellor et al., Biochem. J. 332:281-292, 1998). The presumptive ortholog of PKCλ in *C. elegans*, PKC-3, colocalizes with Par3 and Par6 at the anterior pole of the one-cell embryo (Tabuse et al., supra; Hung et al., Development 126:127-135, 1999). PKC-3 is necessary for establishment of embryonic polarity, and inactivation of PKC-3 leads to mislocalization of the Par genes and a symmetrical first cell division. *Drosophila* possesses only one atypical PKC (DaPKC), which also associates with a Par3-like protein (Bazooka) and is implicated in control of cell polarity (Wordarz et al., supra). DaPKC mutants exhibit disordered epithelial layering, irregular cell shapes, and loss of epithelial cell polarity, believed to be due to defects in cell adhesion. In vertebrate cells, PKCλ and PKCζ both localize to epithelial tight junctions and associate with a Par3-like protein (ASIP) (Joberty et al., Nat. Cell Biol. 2:531-539, 2000; Suzuki et al., J. Cell Biol. 152:1183-1196, 2001; Lin et al., Nat. Cell Biol. 2:540-547, 2000; Izumi et al., J. Cell Biol. 143:95-106, 1998). We therefore examined whether the *has* mutation and concentramide treatment perturb epithelial patterning and tight junctions, focusing upon the retina and the kidney.

The neural retina arises from an epithelial sheet that is bordered by the lens on the basal surface and by a second epithelial sheet (the retinal pigmented epithelium, RPE) on the apical surface (Schmitt et al., J. Comp. Neurol. 344:532-542, 1994). Prior to cell differentiation, the nuclei of the neuroepithelial cells migrate between the apical and basal surfaces of the epithelium. During M-phase, cell nuclei localize to the apical surface, adjacent to the neighboring RPE (Sauer, J. Comp. Neurol. 62:377-405, 1935). Beginning at about 30 hpf, these neuroepithelial cells exit the cell cycle and differentiate into one of seven distinct cell types (Altshuler et al., "Specification of Cell Type in the Vertebrate Retina," *In* Development of the Visual System, Lam et al. (Eds.), The MIT Press, Cambridge, MA 37-58, 1991; Dowling, "The Retina," Belknap Press, Cambridge, MA, 1987). Each cell type then migrates to a specific layer in the retina, resulting in a highly organized, laminar pattern (see Fig. 4A).

The *has* mutation causes disruption of the layering of the neural retina and patchy loss of the RPE (Fig. 4E). These defects resemble those noted previously in zebrafish bearing the mutations *oko meduzy* (*ome*) and *mosaic eyes* (*moe*) (Jensen et al., Development 128:95-105, 2001; Malicki et al., Development 126:1235-1246, 1999). In *has* mutants, the severity of laminar disruption correlates with the position and degree of RPE discontinuity, suggesting that the RPE epithelial defect causes or exacerbates that of the neural retina. This would be concordant with the evidence that a normal RPE is critical to lamination (Raymond et al., Curr. Biol. 5:1286-1295, 1995; Vollmer et al., Neurosci. Lett. 48:191-196, 1984) and the fact that the retinal epithelium of *has* mutants manifests at least one attribute of proper apical-basal polarity in that the majority of the mitotic nuclei localize correctly to the apical surface of the neuroepithelium (Figs. 4B, 4D, and 4F; 89% of M-phase nuclei from *has* embryos localize to the apical surface versus 97% of nuclei from wild-type embryos). As a marker of tight junctions, we examined immunoreactive zonula occludens (ZO-1), an integral tight junction protein, and find it to be mislocalized (Figs. 4G and 4H). Therefore, loss of adhesion between RPE cells may be a cause of retinal mispatterning in *has* mutants. Notably, retinas from concentramide-treated embryos do not exhibit defects in cell polarity (Fig. 4D), RPE continuity, or lamination (Fig. 4C)

The developing kidney is another structure composed of highly polarized epithelial cells. We examined the distribution of apical and basolateral proteins in the kidneys of wild-type, *has*, and concentramide-treated embryos. As in the retina, cell polarity appeared to be largely conserved in *has* kidneys (Figs. 5A-5C). The *has* kidneys did, however, exhibit irregularities in the shapes of epithelial cells and occasional gaps between cells, consistent with a defect in epithelial cell adhesion. We did not observe these defects in embryos exposed to concentramide.

Given the differences between *has* and concentramide-treated embryos with regard to epithelial sheet integrity in the retina and the kidney, it is unlikely that concentramide functions through the same mechanism as the *has* mutation, namely the inactivation of PKCλ. To examine this further, we tested the effect of concentramide on early development of the *C. elegans* embryo. In *C. elegans*, inactivation of the PKCλ ortholog PKC-3 via RNA interference (RNAi) results in the loss of polarized localization of the Par proteins and loss of asymmetry during the first cell division (Tabuse et al., supra). Embryos treated with high concentrations of concentramide retain

proper localization of Par2 to the posterior pole and undergo a normally asymmetric first cell division (Figs. 5D and 5E). Treated embryos exhibit cytokinetic defects and fail to complete development, suggesting that the absence of an asymmetry defect is not due to problems with compound penetration. Therefore, although concentramide treatment and PKCλ inactivation both result in similar heart patterning phenotypes, concentramide does not appear to inactivate zebrafish PKCλ or its nematode ortholog.

The molecular target of concentramide is involved in AP patterning

If the molecular target of concentramide does not affect the continuity of epithelial sheets as PKCλ does, by what sort of process might it influence heart patterning? Treatment with concentramide appears to affect the relative positions of several anatomical structures along the anterior-posterior (AP) axis. For example, the distance between Pax2.1-expressing cells in the eyes and at the midbrain/hindbrain boundary is reduced in concentramide-treated embryos (Figs. 6A-6C). Perhaps more significantly, the cardiac myosin light chain 2 (cmlc2)-expressing cells of the heart field are shifted rostrally in concentramide-treated embryos at the 18-somite stage (Fig. 6D). The distance between the anterior edge of the cmlc2-expressing field and the anterior extreme of the embryo is about 40 percent greater in wild-type embryos (3.1 +/- 0.2 arbitrary units, n=8) than in concentramide-treated embryos (2.2 +/- 0.3 arbitrary units, n=12). The position of the heart field in *has* mutants (3.1 +/- 0.3 arbitrary units, n=12) does not differ significantly from the wild-type position. Therefore, the molecular target of concentramide appears to play a role in AP patterning.

PKCλ and the target of concentramide both influence the fusion order of heart primordia

PKCλ and the molecular target of concentramide appear to act via distinct cellular mechanisms, but modulation of either results in a very similar change in the patterning of the heart. To identify the commonalties between the two mechanisms that allow such similar mispatterning of the heart, we took advantage of the temporal control with which small molecules can modulate biological processes. As described above, we determined that embryos must be treated with concentramide at or prior to the 14-somite stage to cause formation of the ventricle within the atrium. From this observation, we conclude that a critical heart patterning process is initiated shortly after the 14-somite

stage, and perturbation of this process results in the concentric chamber phenotype observed in both *has* and concentramide-treated embryos. This allowed us to focus our search for commonalties between *has* and concentramide-treated embryos to this critical time period.

The generation of the primitive heart tube is accomplished by midline coalescence of the bilateral cardiac primordial sheets. In the zebrafish, this coalescence first generates a single midline cone, with its base on the yolk (Fishman et al., supra; Yelon et al., Dev. Biol. 214:23-37, 1999). Subsequently, the cone tilts to assume a midline A-P orientation with the pre-ventricular end posterior, later to swing anteriorly as yolk is resorbed.

We find that normally the generation of the midline cone does not occur uniformly around the cone's circumference, but rather progresses from posterior to anterior, with posterior regions merging at the 16-somite stage and anterior at the 18-somite stage. This step is perturbed by both concentramide and the *has* mutation. In both *has* mutant embryos and concentramide-treated embryos, there is a failure to merge the posterior ends (Figs. 7A-7C). Even by the 18-somite stage, when the anterior ends of the primordia begin to fuse normally, the posterior ends remain separated in the *has* and concentramide-treated embryos (Figs. 7D-7F). Eventually, the posterior ends do fuse in *has* and concentramide-treated embryos, just before emergence of the concentric chambered heart. Thus, a critical patterning decision occurs at about the 16-somite stage that regulates the fusion order of the anterior and posterior ends of the heart field. This process can be blocked either by inactivation of PKCλ or by modulating the target of concentramide.

Thus, in summary, we have defined a key step in heart formation by its perturbation with a small molecule and a mutation. This step involves the proper alignment of the two cardiac chambers, just as the primitive heart tube assembles. Two perturbants --the small molecule concentramide and the *has* mutation-- both elicit a previously undescribed chamber malalignment, in which the ventricle forms inside of the atrium. This means that establishment of the cardiocyte cell fates is largely accomplished, but the higher order assembly of chamber structure is disrupted.

Experimental Methods

Small molecule treatment

Zebrafish were maintained at 28.5°C as described (Westerfield, "The Zebrafish Book, Guide for the Laboratory Use of Zebrafish (*Danio rerio*)," Univ. of Oregon Press, Eugene 1995). Unless specified otherwise, embryos were treated prior to gastrulation by adding concentramide to the water at a final concentration of 34 nM from a 34 μM stock solution in DMSO.

Whole-mount in situ hybridization and immunohistochemistry

Digoxigenin-labeled antisense RNA probes were generated by *in vitro* transcription for cmlc2 (Yelon et al., supra), vmhc (Yelon et al., supra), and pax2.1 (Krauss et al., Development 113:1193-1206, 1991). *In situ* hybridization was carried out as described (Oxtoby et al., Nucleic Acids Res. 21:1087-1095, 1993). For whole-mount immunohistochemistry, embryos were fixed in 4% paraformaldehyde in phosphate-buffered saline (S46 and 3G8) or 80% methanol, 20% dimethyl sulfoxide (α-ZO-1), permeablized in acetone for 30 minutes at -20°C (3G8), blocked with 5% fetal bovine serum, and incubated with the antibodies S46, 3G8 (Vize et al., Dev. Biol. 171:531-540, 1995), or α-ZO-1. An anti-mouse-horseradish peroxidase conjugate was used as secondary antibody for S46 and 3G8, and an Alexa 488-labeled anti-mouse secondary antibody was used for α-ZO-1 staining.

Histology

Fixed embryos were dehydrated, embedded in plastic (JB-4, Polysciences, Inc.), and sectioned at 2-7 μm. Retinal sections were stained with hematoxylin-eosin or dapi.

Cloning of has

Embryos were separated into mutant and wild-type pools based on phenotypic analysis. Genomic DNA was isolated from individual embryos by incubation in DNA isolation buffer overnight at 50°C (DNA isolation buffer: 10 mM Tris-HCl, pH 8.3; 50 mM KCl; 0.3% Tween-20; 0.3% Nonidet P40; 0.5 mg/ml proteinase K). Proteinase K was inactivated prior to PCR setup by heating samples to 98°C for 10 minutes. PCR

reactions were performed using diluted genomic DNA as described (Knapik et al., Development 123:451-460, 1996).

RNA was isolated (RNeasy columns, Qiagen) from pools of wild-type and mutant embryos to generate cDNA for RT-PCR analysis (SMART RACE cDNA amplification kit, Clontech). Fragments were then subcloned into PCRII-TOPO (Invitrogen). PCR primers were synthesized based on sequence from an EST for PKCλ (fc69h04, GenBank accession# AI883774) and genomic sequence (Genome Systems, BAC clone address 23c14), and used to sequence the entire PKCλ coding region and 3'UTR.

Genomic clones were isolated by PCR analysis of DNA pools from BAC (Genome Systems) and YAC (Research Genetics) libraries using primer sets for the linked markers z11023 and z8451. YAC end sequence was determined as described (Zhong et al., Genomics 48:136-138, 1998). BAC ends were sequenced directly using SP6 and T7 primers, and BACs 53c17, 89i15, and 152p21 were subcloned by shotgun cloning of partial AluI digested fragments into pBluescript. For the complete sequencing of BACs, a hydroshear was used to produce fragments of 2-3kb in length. These fragments were then blunt-end ligated into pGEM5 (Promega) and sequenced using an ABI3700 to generate approximately five-fold coverage. The sequence was assembled using the Phred/Phrap/Consed programs (Gordon et al., Genome Res. 8:195-202, 1998; Ewing et al., Genome Res. 8:186-194, 1998; Ewing et al., Genome Res. 8:175-185, 1998).

Western blotting

Groups of 25 embryos were lysed in 0.5% Triton X100 in phosphate-buffered saline. Lysates were clarified by centrifugation and separted by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis. Western blotting was performed using an α-PKCλ rabbit polyclonal antibody (Santa Cruz Biotechnology, Inc.).

Morpholino injection

An antisense morpholino oligonucleotide of sequence 5'-CTGTCCCGCAGCGTGGGCATTATGG-3' (GeneTools, LLC) was dissolved at a concentration of 100 μM in 1X Danieau's buffer (5 mM Hepes pH 7.6, 58 mM NaCl, 0.7 mM KCl, 0.6 mM $Ca(NO_3)_2$, 0.4 mM $MgSO_4$). One nL of this solution or 1X

Danieau's buffer was injected into each 1-4 cell embryo before allowing the embryos to develop at 28.5°C.

C. elegans development

C. elegans strain KK871 (par-2::GFP) was maintained at 25°C. For each sample, 10-15 adult worms were soaked in 80 µL M9 medium containing 34 µM concentramide, 0.25% dimethyl sulfoxide for 30-60 minutes. Worms were then cut open with a scalpel, and embryos were mounted on 2% agarose pads with coverslips. Embryos were allowed to develop at 25°C before being photographed live.

Other Embodiments

All publications and patent applications mentioned in this specification are herein incorporated by reference to the same extent as if each independent publication or patent application was specifically and individually indicated to be incorporated by reference.

While the invention has been described in connection with specific embodiments thereof, it is to be understood that it is capable of further modifications and this application is intended to cover any variations, uses, or adaptations of the invention following, in general, the principles of the invention and including such departures from the present disclosure that come within known or customary practice within the art to which the invention pertains and can be applied to the essential features hereinbefore set forth, and follows in the scope of the appended claims.

What is claimed is:

Claims

1. A method of determining whether a test subject has, or is at risk of developing, a disease or condition related to Protein Kinase C λ, said method comprising analyzing a nucleic acid molecule of a sample from the test subject to determine whether the test subject has a mutation in a gene encoding said Protein Kinase C λ, wherein the presence of a mutation indicates that said test subject has, or is at risk of developing, a disease or condition related to Protein Kinase C λ.

2. The method of claim 1, wherein said test subject is a mammal.

3. The method of claim 1, wherein said test subject is a human.

4. The method of claim 1, wherein said disease or condition is a disease or condition of the heart or cancer.

5. The method of claim 1, wherein said disease or condition is associated with epithelial-epithelial cell interactions or epithelial cell polarity.

6. The method of claim 1, wherein said mutation results in a carboxyl terminal truncation of Protein Kinase C λ.

7. The method of claim 1, wherein said mutation is the *heart and soul* mutation.

8. A method for identifying a compound that can be used to treat or to prevent a disease or condition of associated with Protein Kinase C λ, said method comprising contacting an organism comprising a mutation in a gene encoding Protein Kinase C λ and having a phenotype characteristic of a disease or condition associated with Protein Kinase C λ with said compound, and determining the effect of said compound on said phenotype, wherein detection of an improvement in said phenotype indicates the identification of a compound that can be used to treat or to prevent said disease or condition.

9. The method of claim 8, wherein said disease or condition associated with Protein Kinase C λ is heart disease.

10. The method of claim 8, wherein said organism is a zebrafish.

11. The method of claim 8, wherein said mutation results in a carboxyl terminal truncation of Protein Kinase C λ.

12. A method of treating or preventing a disease or condition associated with Protein Kinase C λ in a patient, said method comprising administering to said patient a compound identified using the method of claim 8.

13. The method of claim 12, wherein said disease or condition is of the heart.

14. The method of claim 12, wherein said patient has a mutation that results in a carboxyl terminal truncation of Protein Kinase C λ.

15. A method of treating or preventing a disease or condition associated with Protein Kinase C λ in a patient, said method comprising administering to said patient a functional Protein Kinase C λ protein or an expression vector comprising a nucleic acid molecule encoding said protein.

16. A method of treating or preventing a disease or condition associated with Protein Kinase C λ in a patient, said method comprising administering to said patient a compound or molecule that alters the activity or expression of Protein Kinase C λ in said patient.

17. A substantially pure zebrafish Protein Kinase C λ polypeptide.

18. The polypeptide of claim 17, wherein said polypeptide comprises an amino acid sequence that is substantially identical to the amino acid sequence of SEQ ID NO:2.

19. The polypeptide of claim 17, wherein said polypeptide comprises the amino acid sequence of SEQ ID NO:2.

20. A substantially pure polypeptide comprising the sequence of SEQ ID NO:2 and variants thereof comprising sequences that are at least 95% identical to that of SEQ ID NO:2, and which have Protein Kinase C λ activity.

21. An isolated nucleic acid molecule comprising a sequence encoding a zebrafish Protein Kinase C λ polypeptide.

22. The nucleic acid molecule of claim 21, wherein said nucleic acid molecule encodes a polypeptide comprising an amino sequence that is substantially identical to the amino acid sequence of SEQ ID NO:2.

23. The nucleic acid molecule of claim 21, wherein said nucleic acid molecule encodes a polypeptide comprising the amino acid sequence of SEQ ID NO:2.

24. An isolated nucleic acid molecule that specifically hybridizes under high stringency conditions to the complement of the sequence set forth in SEQ ID NO:1, wherein said nucleic acid molecule encodes a protein that has Protein Kinase C λ activity.

25. A vector comprising the nucleic acid molecule of claim 21.

26. A cell comprising the vector of claim 25.

27. A non-human animal having a knockout mutation in one or both alleles encoding a Protein Kinase C λ polypeptide.

28. A cell from the non-human knockout animal of claim 27.

29. A non-human transgenic animal comprising a nucleic acid molecule encoding a mutant Protein Kinase C λ polypeptide.

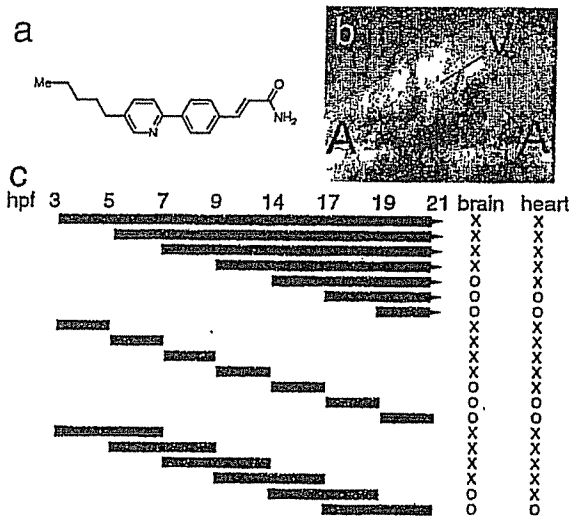
30. The non-human transgenic animal of claim 29, wherein the non-human transgenic animal is a zebrafish.

31. The non-human transgenic animal of claim 29, wherein the non-human transgenic animal comprises the *heart and soul* mutation.

32. An antibody that specifically binds to a Protein Kinase C λ polypeptide.

33. A method of modulating the activity of a Protein Kinase C λ polypeptide in a patient, said method comprising administering to the patient an RNA that stimulates or inhibits this activity.

Fig. 1



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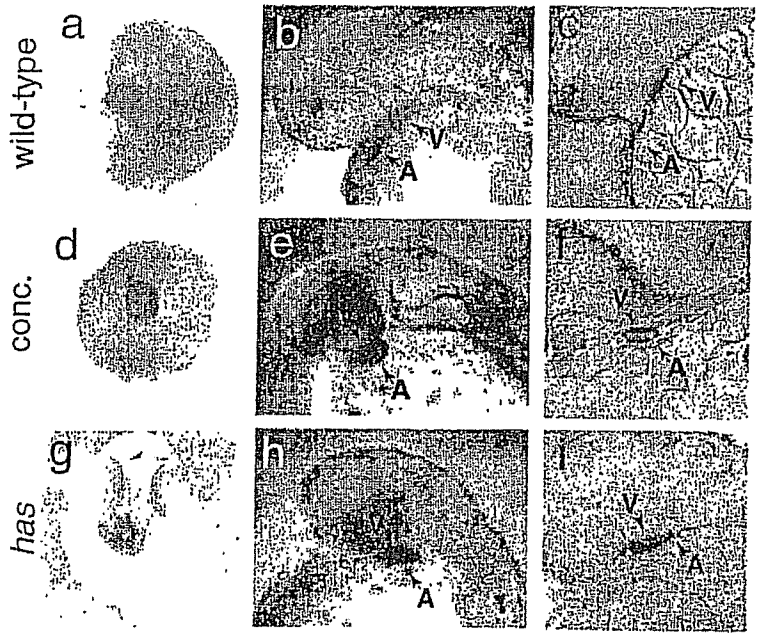

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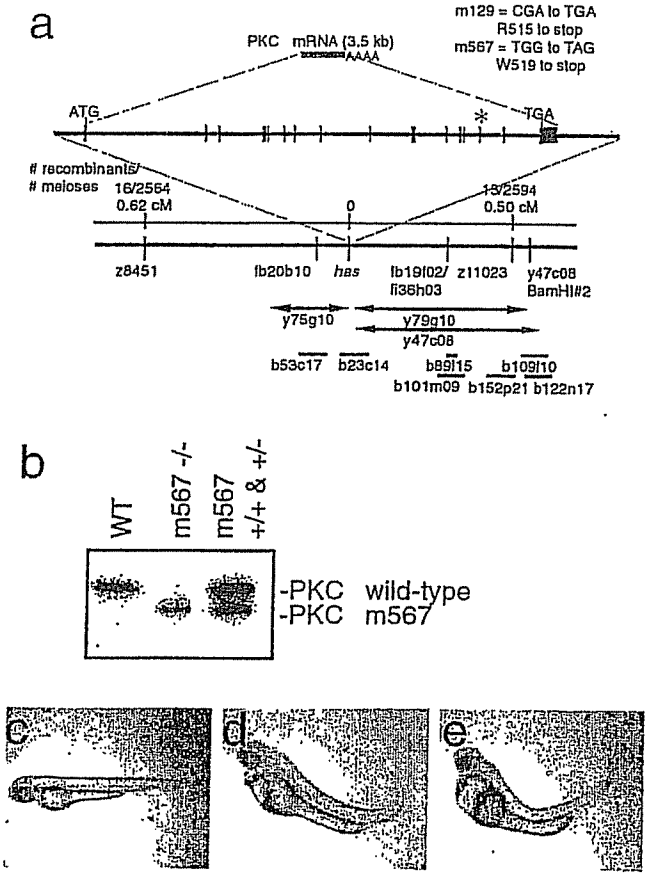
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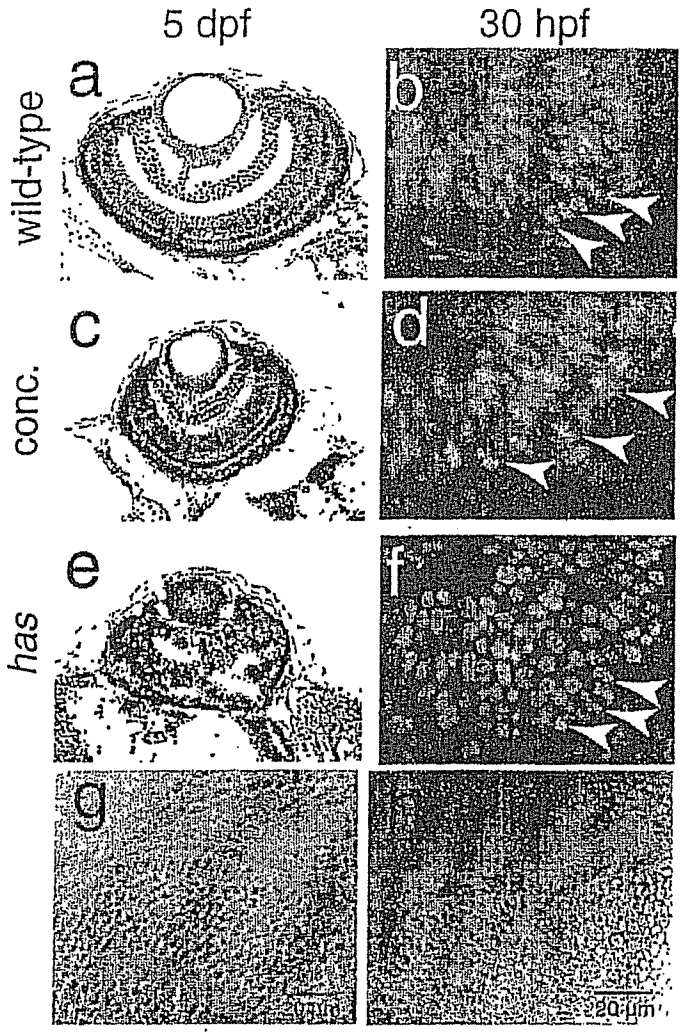

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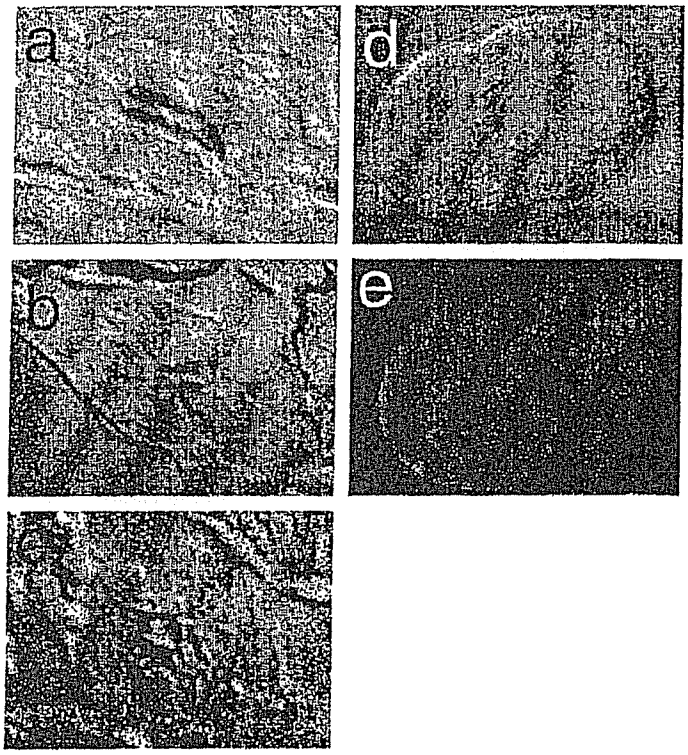

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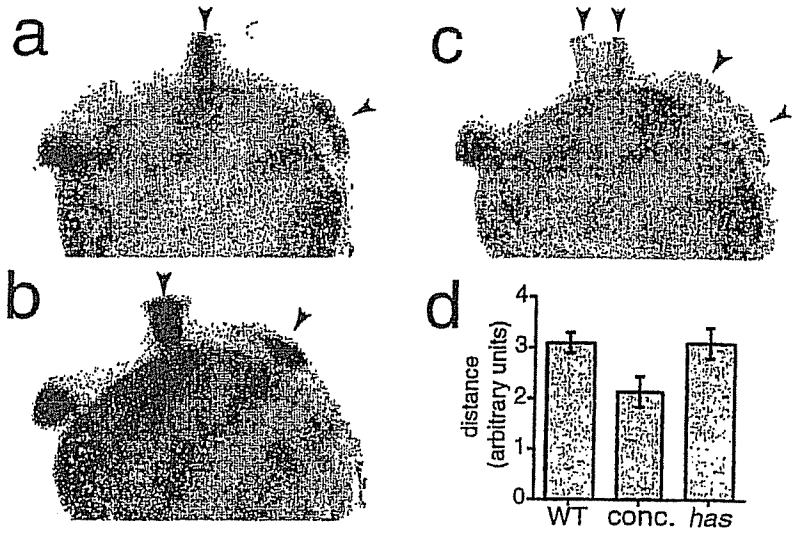

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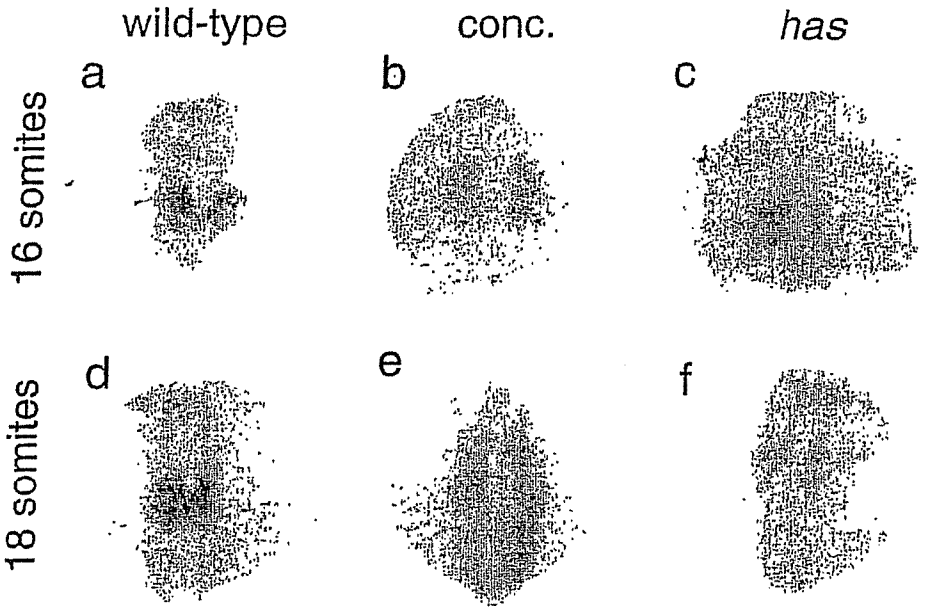

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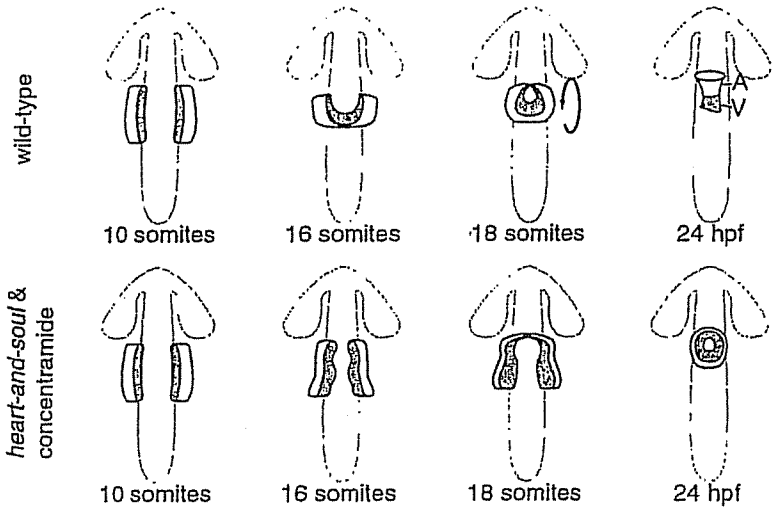

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(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
20 March 2003 (20.03.2003)

PCT

(10) International Publication Number
WO 2003/023048 A3

(51) International Patent Classification[7]: C07K 16/00, 17/00, A01K 67/033, A61K 38/00, 31/70, C07H 21/04, A61K 49/00

(21) International Application Number: PCT/US2002/028410

(22) International Filing Date: 6 September 2002 (06.09.2002)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
60/317,653 6 September 2001 (06.09.2001) US

(63) Related by continuation (CON) or continuation-in-part (CIP) to earlier application:
US 60/317,653 (CIP)
Filed on 6 September 2001 (06.09.2001)

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(72) Inventors; and
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(74) Agent: **MICHAUD, Susan, M.**; Clark & Elbing LLP, 101 Federal Street, Boston, MA 02110 (US).

(81) Designated States *(national)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, OM, PH, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, YU, ZA, ZM, ZW.

(84) Designated States *(regional)*: ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, SK, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:
— *with international search report*

(88) Date of publication of the international search report: 15 January 2004

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

(54) Title: METHODS FOR DIAGNOSING AND TREATING DISEASES AND CONDITIONS ASSOCIATED WITH PROTEIN KINASE Cλ

(57) Abstract: The invention provides methods of diagnosing diseases and conditions associated with PKCλ, methods for identifying compounds that can be used to treat or to prevent such diseases and conditions, and methods of using these compounds to treat or to prevent such diseases and conditions. Also provided in the invention are animal model systems that can be used in screening methods.

WO 2003/023048 A3

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US02/28410

A. CLASSIFICATION OF SUBJECT MATTER

IPC(7) : C07K 16/00; C07K 17/00; A01K 67/033; A61K 38/00; A61K 31/70; C07H 21/04 ; A61K 49/00
US CL : 530/387.1; 530/350; 800/13; 514/2; 514/44; 536/23.1;424/9.2

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
U.S. : 530/387.1; 530/350; 800/13; 514/2; 514/44; 536/23.1;424/9.2

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 2003/0017969 A1 (TENNENBAUM et al.) 23 January 2003 (23.01.2003), page 2, para. 0013-0015; page 3, para. 00143l; page 17, para. 0171.	12,15,16
X,P	HORNE-BADOVINAC, S. et al. Positional cloning of heart and soul reveals multiple roles for PKC lambda in zebrafish organogenesis. Current Biology, October 2001, Vol. 11, pages 1492-1501, see "Gnetic mapping and positional cloning and reference to Genbank Accession #AF390109.	21-25
X --- Y	AKIMOTO et al. A new member oif the third class in the protein kinase C family, PKC-lambda, expressed dominantly in an undifferentiated mouse ebryonal carcinoma call line and also in many tissues and cells. Jour. Biol. Chem., 29 April 1994, Vol. 17, pages 12677-12681, see column 2, lines 8-10 and Figure 1B.	32 --- 27,28, 33
Y	WANG et al. Expression of a dominant negative type II transforming growth factor beta (TGF-beta) receptor in the epidermis of transgenic mice blocks TGF-beta-mediated growth inhibition. PNAS, March 1997, Vol. 94, pages 2386-2391.	29
Y	BANDYOPADHAYA et al. Effects of adenoviral gene trasnfer of wild-type, constitutively active, and kinase-defective protein kinase C-lambda on insulin-stimulated glucose transport in L6 myotubes. Endocrinology, 2000, Vol. 141, No. 11, pages 4120-4127.	29

☒ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

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"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
12 February 2003 (12.02.2003)	31 OCT 2003
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703)305-3230	Authorized officer Valarie Bertoglio Telephone No. 703-308-1234

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